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PANCREATIC ISLET TRANSPLANTATION:
A PROMISING METHOD FOR TREATING THE CHRONIC COMPLICATIONS OF DIABETES MELLITUS

by

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This text is dedicated
to my wife Fay, and our children,
Noelle and Willie

ABSTRACT

Current methods of insulin therapy, however stringent, have not prevented the microvascular complications of diabetes mellitus. New methods of therapy are required. Transplantation of fresh or cryopreserved islets of Langerhans can render diabetic rats of the same inbred strain clinically normal and appears to prevent or reverse the microvascular complications. The purpose of this thesis is to show that it is feasible, in large mammals, to prepare grafts of pancreatic islets, isograft them into the spleen as fresh or frozen-thawed tissue, and preserve their metabolic efficiency.

Thirty-one dogs were rendered diabetic by total pancreatectomy. The pancreatic ducts were perfused with collagenase to prepare a tissue suspension which was isografted into the spleen (mean graft volume was 10 ml, containing 24% of the B-cell mass/pancreas). Apancreatic controls ($n=4$) survived 10 ± 3 days; mean fasting blood glucose (BG) was 343 mg/dl terminally. In 13 dogs, the tissue was implanted by reflux into terminal splenic veins: BG was $\leq$ 150 mg/dl for up to 5 months ($n=6$). During glucose tolerance testing 1 week pre- and 1 mo and 2-3 mo post implant, mean values were respectively: K (decline in glucose concentration, %/min) 3.4, 1.4 and 1.5, peak insulin (μU/ml) 50, 12 and 11. Post implant insulin levels in splenic veins peaked at 1 min (55) and 60 min (77). Diabetes ensued after splenectomy. Splenic histology showed prominent islets. In 5 dogs, the tissue was injected into the splenic pulp; BG rose to $\geq$ 250 mg/dl (compared with initial series, $p < 0.001$) and remained elevated. In 5 dogs, when tissue was equilibrated in the

cryoprotectant at 0°C, then frozen to -196° for 48 h, diabetes ensued after implantation; when 25°C was used for equilibration, BG was $\leq$ 125 mg/dl for 3 mo (n=2). Collagenase perfusion of a large mammal pancreas via the ducts provides sufficient viable islets to induce prolonged normoglycemia (5 months). Reflux of pancreatic fragments into splenic veins appears more efficient than intrapulp implantation. The graft function does not appear to deteriorate during prolonged follow-up. Insulin secretion from the isografted intrasplenic islets is biphasic in response to glucose challenge. After cryopreservation with a higher temperature during exposure to the cryoprotectant, frozen-thawed isografts induce normoglycemia.

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I INTRODUCTION

Diabetes mellitus is a common chronic disease with severe long-term complications. Its incidence is increasing by 6% per year, a rate that will result in doubling of the number of diabetics in the population every 15 years (1).

The complications of diabetes, which include retinopathy, neuropathy, macro-angiopathy, and micro-angiopathy, give rise to significant morbidity and mortality. The 1975 report of the National Commission on Diabetes to the United States Congress (1) included the following statistics.

- Patients with diabetes are 25 times more prone to blindness than those without diabetes.
- Diabetic retinopathy is the second-commonest cause of blindness overall and the leading cause of new cases of blindness.
- Compared with the general population, diabetics are 17 times more prone to renal disease, 5 times more prone to gangrene of the extremities, and twice as prone to heart disease.

The diabetes-related mortality figures are similarly high:

- The life-expectancy of diabetic patients is two-thirds that of the general population (1).
- Diabetes is estimated to be the fifth-commonest cause of death in the USA - and it has been suggested that inclusion of all contributory and underlying causes of death (diabetes being infrequently recorded as a cause), and correction for the large number of undiagnosed cases of diabetes, might indicate diabetes

mellitus as the third-commonest cause of death in the USA (1).

- Three-quarters of North America's diabetics will die of atherosclerotic disease, in contrast to one-third of the general population (2).

Why do these devastating complications occur, despite the availability of insulin for more than 60 years? Evidence indicates that the micro-angiopathic lesions are secondary to persistent hyperglycemia - and current methods of insulin therapy do not reliably prevent wide fluctuations in blood-glucose levels even when so-called 'tight control' is maintained (3).

- First, prospective studies have shown a relationship between the height of blood-glucose levels and the severity of complications (4-7).
- Second, the complications occur in patients with diabetes resulting from total pancreatectomy, and hemochromatosis (8-9). This rules out a purely genetic predisposition of the micro-angiopathy.
- Third, after the induction of diabetes in animals, micro-angiopathic lesions histologically similar to those in humans develop and their severity reflects the adequacy of metabolic control (10).

These findings have intensified the search for better methods to maintain normoglycemia.

New Methods of Treatment

New methods of treatment can be grouped into two categories: (a) systems designed to administer insulin continuously; and (b) pancreatic transplantation, of the whole organ, islet-containing tissue, or isolated islets.

Insulin-secreting Devices

Implantable devices that secrete insulin (artificial beta { B } cells) in response to the continuous fluctuations in blood-glucose level are expensive, and they involve cumbersome, complicated technology. Biocompatibility of materials to sense glucose fluctuations, compute insulin requirements, and secrete an appropriate amount of insulin is a problem.

Furthermore, the peripheral route of insulin delivery does not offer the physiological advantages of portal insulin delivery in maintaining fuel homeostasis (11). If normoglycemia is achieved with open-loop insulin pumps that administer insulin systemically, peripheral hyperinsulinemia results (12). This may have undesirable consequences on fuel other than glucose; also, it seems that hyperinsulinemia is a significant factor in the development of atherogenesis in persons who have non-insulin-dependent diabetes mellitus (NIDDM) (2). Certain insulin-delivery systems create problems with hypoglycemic reactions, especially in persons with insulin-dependent diabetes mellitus (IDDM) (12).

In summary, insulin-delivery systems are unlikely to be as efficient as functioning B cells.

Transplantation of Pancreatic Tissue

"Total endocrine replacement therapy", a phrase coined by Dr. D. Sutherland, is the objective of pancreatic transplantation. In other words, transplanted functioning B cells should permit precise glucoregulation and fuel homeostasis, not only in response to ingested carbohydrates but also in dynamic states such as stress, fasting, exercise and injury.

B cells are integrators of multiple stimulatory and inhibitory signals arising both within and beyond the islets of Langerhans. Internal signals refer to interactions between A cells (glucagon-producing), D cells (somatostatin-producing), and B cells (insulin-producing). For example, somatostatin may inhibit both insulin and glucagon secretion locally, glucagon may stimulate the secretion of both insulin and somatostatin, and insulin may inhibit the release of glucagon and somatostatin (13). Tight junctions, termed 'gap junctions', and constant non-random distribution of cells within the islets, provide histologic evidence of the importance of these internal signals (14).
External signals that stimulate secretion include glucose, amino acids, fatty acids, beta-adrenergic catecholamines, acetylcholine, and intestinal peptide hormones. Those that inhibit insulin secretion include prostaglandin-E (14).

Results of pancreatic transplatation in animals have proved that microvascular lesions can be prevented by maintaining strict normoglycemia (15-17). Also, strict control of the blood-glucose level reverses the increased aggregation and decreased deformability of the erythrocytes, the increased aggregation of platelets, and the altera-

tions in plasma proteins, that may alter blood viscosity and flow and thus favor the development of atherogenesis in diabetics (2).

But strict metabolic control goes beyond simply controlling the blood-glucose level. An increased growth-hormone level, implicated in the pathogenesis of diabetic retinopathy, has been normalized in persons who have the insulin-dependent disease by the implantation of artificial B cells that secrete insulin continuously (18,19). Hyperglucagonemia, by causing gluconeogenesis, is responsible for full expression of uncontrolled diabetes and its attendant complications. This, too, is suppressed during fasting and after meals, by artificial B cells, and in animals by pancreatic transplantation (20,21). Structural renal abnormalities, namely thickening of the glomerular mesangium and basement membrane, result in increased rates of glomerular filtration and albumin excretion. Strict control with insulin normalizes both of these functions, and islet transplantation in animals reverses the structural abnormalities (17,22). Somatostatin is an inhibitor of insulin release. The number of somatostatin-producing D cells is increased in patients who have insulin-dependent diabetes, and somatostatin is secreted in increased amounts in experimental diabetes. Pancreatic transplantation reverses increased somatostatin in diabetic rats (23). Cyclic GMP, possibly a second messenger for insulin action on the liver to control hepatic gluconeogenesis, is increased in diabetic rats but reverts to normal after islet transplantation (24).

First suggested by Ssobelew in 1902 (25) as a treatment for diabetes mellitus, pancreatic transplantation is of two types: immediately vascularized whole or segmental pancreas grafts, and free grafts of

islets of Langerhans. The first experimental pancreatic transplants were whole-organ allografts (26), attempted in the late 1920s by Gayet and Gaillaume and by Houssay, Lewis, and Foglia. The results of these experiments were discouraging, and interest soon shifted to therapy with insulin, discovered by Banting and Best in 1921.

Transplantation thus entered a relatively dormant phase, while medical therapy forged ahead. However, as diabetics lived longer, complications of the disease became apparent, and this led to a resurgence of interest in pancreatic transplantation.

In the mid 1960s, Kelly, Lillihei and associates began trials of human transplants with whole-organ grafts. Though these grafts proved to be metabolically efficient, technical complications and allograft rejection denied long-term success: the problems, coupled with immunosuppression of a host who had multisystem disease, gave rise to very high morbidity rates and mortality. Modifications of the procedure for transplanting the whole organ or segments were introduced; but, more importantly, the concept arose of transplanting free grafts of islets of Langerhans. This was primarily to avoid the dangerous consequences of inadequately controlled exocrine pancreatic secretions.

Experimental islet transplants have now been studied for about 20 years, and signficiant advances have been made. It is these grafts that have demonstrated the ability to prevent or reverse diabetic microangiopathy in small animals. However, although the problems encountered with exocrine secretions have been less than with whole-organ grafts, islet transplantation has enjoyed little success clinically.

Appraisal of the experience in large mammals provokes several

questions. First, is allograft rejection as much a problem as with whole-organ grafts? In fact, it has been suggested that free grafts of islets may be *more* immunogenic (27). Second what is the yield of viable islets from a single donor pancreas? Current isolation techniques are not very satisfactory, the yield being less than 10% of the original islet-cell mass of the gland; this often precludes successful transplantation (28). Third, what is the optimal transplant site and transplantation method? Portal drainage of the graft site is desirable, to ensure that high insulin concentrations reach the liver during glucoregulation; the liver and spleen satisfy this requirement (29). Yet is has not been possible to establish the optimal clinical site. Furthermore, islet-containing tissue that is contaminated with exocrine enzymes can produce disseminated intravascular coagulation and portal hypertension if it embolizes into the liver (30). Fourth, how can harvested islets be stored, to allow tissue-typing and for shipment to the location of recipients? Short-term culture, cold storage, and cryopreservation have been studied (31), but the role of each has not been clearly established. Finally, if sufficient islet tissue could be harvested, would it be metabolically efficient in maintaining fuel homeostasis?

The criteria for successful islet transplantation have been summarized by Kretchsmer *et al*. and Brooks (32,36) as follows.

> Successful transplantation of pancreatic islet tissue depends upon the same factors essential for transplantation of other endocrine tissue as a free graft: (1) tissue fragments must be small enough to survive by nutrient diffusion until neovascularization occurs. (2) The site of implantation must be well vascularized. (3) A critical mass of tissue must be transplanted. A unique and critically important requirement for islet transplantation relates to the need to protect the transplanted tissue and the host from the destructive effects of pancreatic exocrine enzymes.

Each of these criteria will be appraised, with a view to supporting the contention that islet transplantation will be the method of choice for treating the complications of diabetes mellitus and perhaps, in time, preventing them. A review of the experience with islet transplantation is now in order, as this defines the problems and future clinical applications.

II REVIEW OF THE LITERATURE

Historical Perspectives

Pancreatic islets were first described by Paul Langerhans (1847-1888) in his doctoral thesis of 1869. He had observed, at low magnification, tiny yellow spots (0.1 to 0.2 mm in diameter) in rabbit pancreas, and found them to be composed of small, regular, polygonal cells, 9 to 12 µm in diameter. Zellhaufen (heap of cells) was the term he applied, in confessing ignorance of their function (33).

It was not until late in the nineteenth century that diabetes mellitus and the pancreas began to be linked in the developing concept of the disease (34). In 1889, Minkowski discovered accidentally in dogs that symptoms of diabetes developed after he had performed total pancreatectomy. This finding was fortunate, as J.C. Brunner (1653-1727), performing similar experiments almost two centuries earlier, had noted thirst and polyuria after total pancreatectomy in dogs but had not made the association with diabetes. Minkowski reported his findings with von Mering, and together they are credited with establishing the pancreas as an organ of internal secretion and the one involved in diabetes. Minkowski even proposed that an extract of pulverized pancreas be injected to eradicate the symptoms of diabetes (34).

The focus was narrowed to the islets by E.G. Languesse, in 1893. He was the first to suspect that they contributed the internal secretion of the pancreas, and it was he who coined the phrase "island of Langerhans" (33).

In 1894, Williamson expressed the idea of transplanting the pancreas from animals to diabetics (34), and shortly after this L.W.

Ssobelew (1876-1919), in Petrograd {Leningrad}, extended Minkowski's work. He ligated the pancreatic duct in dogs, cats and rabbits, causing exocrine parenchymal atrophy, but noted that the islets remained functional and diabetes mellitus did not occur. Having established that the islets and not the acinar cells were necessary to control fuel metabolism, Ssobelew proposed transplantation as a method for using the islets' internal secretion to treat diabetes. Translation of a statement by Ssobelew in 1902 reads as follows (25):

> By ligating the pancreatic duct, we now have a means of isolating the islands anatomically and of studying their chemical properties freed from the digestive fervents. This anatomical isolation will permit rational testing of organotherapy for diabetes.

Simultaneously, E.L. Opie, at Johns Hopkins, reached the same conclusions, based on his observations of pathological changes in islet tissue of diabetics at necropsy.

The scope of the work by Ssobelew and Opie was not fully appreciated at the time, and research centered on other aspects during the first two decades of the 1900s. Histologic studies by Lane led to his description in 1907 of type-A and type-B cells in the islets, and in 1915 Homans stated that B cells probably produce insulin. Numerous investigators tried to cure diabetes by injecting pancreatic extracts, but toxic symptoms precluded success. In general, the concept that the islets produced a hormone responsible for fuel homeostasis was being doubted.

In November 1920, Dr. Frederick Banting, a surgeon, was stimulated by an article in the periodical Surgery, Gynecology and Obstetrics (34,35) to review all the work on isolation of an antidiabetes hormone. In the

spring of 1921 he was joined by Dr. C.H. Best at the University of Toronto. Working together, Banting and Best revived the theory that blockage of the pancreatic duct could anatomically isolate pancreatic islets. They did not attempt islet transplantation; instead, they applied the concept to extraction of the islets' secretion, and this they obtained in relatively pure form. Banting and Best found that this substance ameliorated diabetes in depancreatized dogs and termed their discovery "isletin"; the name was changed to 'insulin' at the insistence of J.J. McLeod, head of the laboratory in which they worked.

The era of insulin therapy had dawned. In 1936, H.C. Hagedorn, from Copenhagen, discovered that monoprotamines could delay the absorption of insulin and, thus, that the combination of these with insulin provided longer control of the blood-glucose level. Insulin therapy was further improved by the addition of zinc to protamine insulin, and in World War II and the 1950s the oral sulfonureas were discovered. These developments attracted attention away from pancreatic transplantation for more than 30 years.

As insulin therapy allowed diabetics to live longer, however, chronic devastating complications became apparent, reviving the interest in transplantation - to prevent rather than treat the complications. Two factors helped to stimulate interest in transplanting the islets. First, early whole-pancreas or pancreatic-segment transplantations were complicated by graft pancreatitis, autodigestion, problems with exocrine secretions, vascular thrombosis, and rejection (26). Second, in general, immediate vascularization is not essential for an endocrine-tissue transplant - an important advantage over whole-organ grafts.

The stage was now set for experimental islet transplantation in animals.

A Review of Techniques for Preparing Free Grafts of Islet Tissue (Animal Studies)

Early attempts at islet transplantation were performed in dogs. In 1935, Selle transplanted cultured fragments of fetal and duct-ligated pancreases into the subcutaneous tissue (37). In 1960 Carnevali and Remine and their associates autotransplanted slices of duct-ligated pancreas subcutaneously (38). These attempts were unsuccessful. However, the studies highlighted practical hurdles to be overcome in separating the tiny islands, constituting a mere 1-2% of the pancreas, from the exocrine component. Could maximal yields of purified, still-viable islets be obtained? - this question is still unanswered. Indeed, other problems (such as optimal transplant site, metabolic efficiency, graft storage and immunologic rejection) have received only superficial attention because of the insufficiency of islets harvested.

Tissue has been taken from three sources (adult, neonatal, and fetal donors) in the quest for higher yields of purified, viable islets, and three aspects have been studied: isolation of purified islets, dispersion of islet tissue without islet isolation, and isolation of islet cells.

1. Adult Donor Tissue

a. Isolated islets

Injection of neutral red dyes that stain islets selectively and free-

hand microdissection of islets have been practised for many years (39). But the practical application of these techniques for isolating sufficient numbers of islets for transplantation is limited. A major advance in islet isolation was in 1965 when Moskalewski used collagenase, an enzyme complex derived from Clostridium histolyticum, to separate intact islets from chopped guinea-pig pancreas (40). In 1967, Lacy and Kostianovsky determined that mechanical disruption of the pancreas by ductal injection of a salt solution increased enzymatic contact with the exocrine tissue and thus increased the yield of purified islets (41), but still it was necessary to separate the endocrine cells from the other components of pancreas. Initially, hand-picking was done, but this is tedious and impractical for large-scale isolation. Ficoll, a sucrose polymer of high molecular weight, was found useful for separating islets. Termed discontinuous density-gradient separation, this method was devised by Lindall and associates (42). Ficoll solutions of different concentrations are combined with digested, washed pancreatic fragments, then centrifuged. The islet-rich tissue concentrates in certain layers of the ficoll solution, allowing faster harvest of pure-islet tissue.

In 1972, Ballinger and lacy, using these techniques, reported the first sustained success of transplanted isolated islets from adult rats (43). They isolated about 200 islets from each pancreas (a rat pancreas contains 5000 to 10,000 islets) and implanted 400-600 intraperitoneally in rats with streptozotocin-induced diabetes. Hyperglycemia, polyuria, glycosuria and weight loss were reversed.

This landmark in transplanting purified islets set two standards.

First, 5-10% of islets were isolated from single pancreases. Second, for successful transplantation, multiple donors were required to reverse diabetes in a single recipient. A further advance was Kemp's demonstration (44) that, for a given number of islets, the intraportal route of transplantation was more effective. Following this, collagenase digestion, ficoll purification and intraportal transplantation became the standard islet isolation and transplantation techniques, and these were reproduced in several research laboratories (44,45).

These techniques for isolation have been extended to mouse, dog, pig, monkey and human pancreases. However, even lower percentage yields are obtained from large-mammal pancreas, because of its compactness, and minimal effects on diabetes have been observed after the implantation of islets in the peritoneal cavity of depancreatized pigs (46) or infusion into the portal veins of monkeys made diabetic by streptozotocin and partial pancreatectomy (47).

Lorentz reported good effects of isolated islets transplanted in dogs (48), but there have been no reports of long-term effects of islet transplantation in dogs made diabetic by total pancreatectomy, a most severe and reliable model of diabetes.

Thus, new approaches were examined to increase islet yields from 5-10% of the B-cell mass from single pancreases. One approach was to deplete the exocrine enzyme content of the donor pancreas by administering pilocarpine beforehand; in rats, this doubled both the islet yield and the exocrine attrition as reflected by insulin and amylase content (49). Downing modified the method to distend the venous system of the pancreas rather than the ducts (71). Distention of the veins disrupts

the tissue within the pancreatic lobules (intralobular) rather than between them (interlobular), to break down barrier presented by the compactness of the mammalian pancreas. With ductal distention, the fibrous tissue enables the acini to resist rupture, so that the ducts rupture first and disruption occurs along the interlobular planes in which they ramify; whereas venous distention permits intralobular disruption, because the islets are richly endowed with venous sinusoids. Applying these principles, Downing quadrupled yields of canine islets from single pancreases; however, this seemed to damage islets, as evidenced by impaired insulin release (71). Meanwhile, Scharp had devised a filtration chamber that filters islets from the digestate immediately they are released, thereby limiting islet digestion (39).

Even with these advances, significant problems remain in isolating intact, viable islets. These include warm ischemia and autolysis in donor tissue, optimal time for mincing, optimal exposure to collagenase, and purification techniques.

Warm ischemia harms all components of donor tissue. In the pancreas, this is highly critical because damaged exocrine cells release noxious digestive enzymes that are most active at $37^{o}C$; this causes autolysis, compounding the ill effects of warm ischemia on the endocrine component. Schulak (51), who evaluated warm ischemia in whole-organ grafts in rats, found that the endocrine pancreas could tolerate up to 90 minutes of warm ischemia without ill effects on histology or graft function as assessed by intravenous GTT. However, this may not apply to free grafts submerged in collagenase and tryptic enzymes. Scharp states that the ability to isolate islets from mammalian pancreas is greatly

reduced within minutes of death of the donor (39). Thus, tolerance of warm ischemia may be briefer during isolation of free islet grafts than with whole organs.

Mincing of the pancreas results in marked attrition of B cells as reflected by insulin recovery in the grafts: Kretschmer (32) reported 50% reduction in tissue insulin after use of a mechanical mincer. Gentler methods of islet separation are required.

A major problem is the provision of optimal conditions for collagenase digestion. Different batches of collagenase give completely different results (as can different concentrations of the enzymes), because effectiveness of different lots has not been standardized. Further, the properties essential for digestion have not been established for collagenase, a mixture of enzymes including collagenase, clostripan, trypsin, neutral nonspecific protease and other unspecified enzymes (52). Thus, at present, the efficacy of collagenase lots can be assessed only by trial in laboratory animals - and this assessment is subjective, optimal digestion being described in terms such as 'mucoid' and 'softened'. Futhermore, as the amount of each lot of collagenase is limited, there is always the frustrating prospect of starting with a new lot of collagenase in the middle of an experimental group. All this adds up to much time and expense and the possibility of obtaining non-comparable results.

There are alternative methods of digestion, mainly for production of single cell suspensions rather than isolated islets. Trypsin is one alternative enzyme. A component of collagenase, trypsin has been used as a primary digestive enzyme (28) during isolation. But trypsin

digestion can impair insulin release from isolated islet cells (53). Dispase, another proteolytic enzyme from *Bacillis polymyxa*, has been used by Ono for dissociating pancreas into single cells (53). When combined with EDTA, it aided isolation and preserved both the cells' histologic characteristics and their ability to release insulin *in vitro*.

Is collagenase necessary? Hinshaw *et al* (54), harvested islets from small-animal pancreas by hand-pressing the organ through a 200-280-μ sieve; they did not use collagenase to digest the glands. Purification was not quantified, but the islets responded to glucose challenge *in vitro*, and the yields of islets or islet-fragments per pancreas were 1.8×10^6 in rabbits and 0.7×10^6 in rats.

With large-mammal pancreas, purification techniques, well developed with ficoll gradient separation in rats, yield prohibitively high acinar contamination (39). Further, to circumvent the rejection problem, Finke, Lacy and Ono (55) devised a method to purify islets of lymphoid cells. Islets accumulate phenol red, permitting differentiation from contaminants when examined under the dissecting microscope with a low-intensity green-light filter. But the technique is still tedious and time-consuming.

Recently, a method of purification has been introduced that is based upon a differential susceptibility of islets and exocrine tissue to cold: Hinshaw used chilling and differential centrifugation to isolate islets and islet-containing tissue from rabbit and rat pancreas (54).

b. Dispersed fragments

Because the isolation techniques precluded collection of more than 5-10% of islets from a single pancreas, implants consisting of islets dispersed within fragments of exocrine tissue were proposed. This was first demonstrated in mice by Kramp *et al* (56). The pancreatic fragments had been rendered enzyme-poor by chronic duct ligation in the prospective donors. However, 3 or 4 pancreases were needed to provide sufficient islets to reverse diabetes in a single recipient. Mehigan (57), who autotransplanted pancreas fragments in dogs, found that his methods for islet preparation (which were effective with normal glands) were ineffective with duct-ligated pancreas. He attributed this to the fibrosis in the latter: scar tissue had surrounded the islets, limiting their vascularization so that they became ischemic and died.

Payne (58), who transplanted dispersed fragments in rats, first depleted the exocrine tissue enzymes with DL-ethionine, which enabled him to recover 40% of the islet mass. He reported amelioration of diabetes in a single recipient with less than one donor pancreas. However, chronic administration of DL-ethionine would not be clinically practicable.

In 1976, Mirkovitch and Campiche (59) showed that sufficient islet tissue to prevent diabetes could be harvested from individual dog pancreases, by completely eliminating purification. Total pancreatectomy was performed; the horizontal part of the pancreas was minced, then digested with collagenase, and the tissue fragments were implanted into the splenic pulp via splenic veins. Results of glucose-tolerance testing postoperatively were similar to those obtained pre-operatively,

and diabetes developed after splenectomy.

Other investigators have reproduced and elaborated upon these experiments. Although the single-donor/single-recipient objective was realized in a large mammal, problems that had plagued free-graft isolation resurfaced. Islet yields were marginally elevated: for example, Kretschmer (29), who dispersed entire canine pancreases and used optimal conditions for graft survival, recovered only 8% of the islet-cell mass. Collagenase was shown to be essential for tissue dispersion (60); revealing the critical balance between tissue dispersal by collagenase and the preservation of sufficient islet-tissue. Mehigan demonstrated the importance of different lots of collagenase in determining outcome (61). Mechanical mincing of the tissue results in 50% loss of the B-cell mass (32), and methods for depleting the exocrine tissue in donor pancreas (DL-ethionine and duct-ligation) are impractical for eventual clinical use.

The optimal fragment size to permit nutrient diffusion until neo-vascularization occurs has not been defined. In the original canine experiments by Mirkovitch and Campiche (59), fragments of 2-4 mm were used successfully, whereas Mehigan (61) found that large particles (passage through 15-gauge needles) were superior to smaller (16-gauge) particles in producing normoglycemia. However, the conclusion that large particle size is advantageous is not necessarily correct, as the smaller particles may have been damaged by excess mincing. In experiments with adult rats, Toledo-Pereyra found a diameter less than 1 mm optimal whereas size > 2 mm denied success (62). Recently, the use of

sieves with mesh of 200-280 μ (54) and 400 μ (28) have confirmed the usefulness of small particle size.

Collagenase ductal perfusion, recently devised for isolating canine islets, is reported to average 57% recovery of the B-cell mass; autotransplantation resulted in normoglycemia in 5 of 7 dogs (28). This technique observes critical factors, including the following. Perfusion of collagenase through the ducts, by creating intralobular distension, selectively digests the exocrine tissue. The vulnerable islets are not immersed in collagenase. The faster digestion shortens warm ischemia. Disruption is gentler and briefer, and purification is obviated by collecting dispersed fragments.

In summary, experiments in large mammals show that pancreatic tissue dispersed by gentle 'mincing' and collagenase digestion can reverse diabetes mellitus. However, eliminating purification increases the yield to only just enough islet tissue from one donor for the transplant to be effective <u>if</u> all of the islets are viable - but not all <u>are</u> viable, and even fewer survive in the transplant site. Thus, these marginal yields are not enough.

c. <u>Islet-cell transplantation</u>

In this approach, pancreases are completely dissociated to populations of single cells and then the endocrine components are reaggregated into 'pseudo-islets'. This method is based on two factors. First, cells placed in rotational culture selectively aggregate with cells similar to themselves (63). Second, methods for preparing single islet cells have been standardized (53). Scharp and associates have combined

these techniques to aggregate canine and pig endocrine cells into pseudo-islets (39,64): initial aggregation occurs within hours, and firm, solid aggregates are complete at 4 days. Histologic studies with immunocytochemistry have shown the A and B cells at the periphery of the aggregates, and D and PP cells more central. Contamination with exocrine cells has averaged 10-30% but the pseudo-islets secrete insulin in response to glucose stimulation *in vitro*. Studies of islet yields and transplantation experiments using these techniques are under way.

Pure populations of A, B, and D rat islet cells have been obtained (63), separated from islet-containing tissue by centrifugal elutriation based upon differences in their sedimentation velocity. The smaller cells (A and D) layer out as one population, and the larger B cells as another. This method, which is speedy, results in about 75% recovery of single islet cells with a high rate of viability, structural integrity, and secretory responsiveness.

2. Neonatal Pancreatic Tissue

a. Dispersed fragments

In neonatal pancreas, the islet volume and their insulin content are high, but the acinar volume and their enzyme content are low, in comparison with adult pancreas. Because of these properties, neonatal rat and mouse pancreas can be minced, dispersed with collagenase, and transplanted, without islet isolation. Indeed, the first demonstration that islet isolation was unnecessary for successful transplantation was with neonatal donor tissue in mice and rats (65,67). The transplants in rats required 20-35 donor pancreases per recipient when implanted

intraperitoneally; when the portal route was used, only one donor organ was required. However, several weeks elapsed before normoglycemia occurred. The B-cell mass of one neonatal rat pancreas (6% of that in an adult rat pancreas) appeared to increase after transplantation; histologically, acinar elements disappeared and mitotic figures were visable.

The major problem with dispersed neonatal pancreases appeared to be low yields of endocrine tissue, necessitating multiple donors and taking a long time to ameliorate diabetes. They did not offer any advantage over adult pancreas donor tissue.

b. Isolated islets

Because of the high relative volume of islet tissue in neonatal tissue, it has not proved worthwhile to develop islet isolation.

c. Islet-cell transplantation

Pseudo-islets have been prepared from pancreases of neonatal pigs (64).

Britt *et al* (64) dissociated the pancreases of 1 to 3 day-old pigs into single cells then reaggregated these in rotational culture. At 7 days, aggregates of cells 0.05-0.2 mm in diameter had formed, containing acinar cells (15-20%), A, B (50%), D and PP cells. Viability *in vitro* was established by demonstrating insulin secretion in response to glucose stimulation. However, transplantation was not attempted, and the possibility remained that altered morphology of the islets may have altered efficacy of fuel regulation *in vivo*.

In summary, neonatal tissue offers the advantage of high yield of

endocrine tissue, thus minimizing graft preparation. Further, it may have a capacity for cell replication. However, it does not appear to offer any advantage over adult tissue, because of the low yield of endocrine tissue, the need for multiple donors, and prolonged time to ameliorate diabetes. Newer techniques of isolating neonatal pseudo-islets have not been tested in vivo.

3. Graft Preparation from Fetal Pancreas

Fetal pancreas, like neonatal tissue, has a favorable ratio of high islet volume to (low) exocrine components. It has the further advantage of replication and differentiation after transplantation.

a. Free grafts

Fetal islets can be purified by culturing pancreases that have been dispersed by collagenase or mincing alone. These preparations have not been tested by transplantation for ability to reverse diabetes; however, they do survive, albeit briefly, in non-diabetic hosts (66).

b. Dispersed tissue

The first report of use of these preparations appeared in 1952 (67). Minced fetal tissue from mice was transplanted intraocularly or subcutaneously in syngeneic alloxan-diabetic mice; there was histologic evidence of graft survival.

The simplest method is to transplant an entire pancreas from a fetus, without vascular anastomosis in the recipient; the endocrine cells replicate after transplantation. In their studies in rats, Brown et al (68) showed that by 2-8 months a single fetal pancreas

attained 22% of the endocrine function of an adult pancreas. McEvoy (69), who transplanted pancreases of 18-day post-coital fetal rats to the renal subcapsular site, showed an 8-fold increase in insulin content when the recipients were killed. Insulin therapy during the immediate post-transplant period was crucial to improving B-cell replication. Brown _et_ _al_ also found that function was best preserved by placing a whole fetal pancreas beneath the renal capsule of a syngeneic host, allowing this 3 weeks to mature, then transplanting the combined graft into the host (70).

In summary, fetal tissue replicates after transplantation, the amount of tissue required is less than with neonatal tissue, and the purity of the endocrine component minimizes the amount of graft preparation. However, there is a long period of latency - even weeks - before the graft ameliorates diabetes. Composite grafts, that circumvent this problem, are complicated by the multidonor immunogenicities, at present precluding use in large mammals.

4. Summary

Islet-tissue can be harvested from three types of donors: fetal, neonatal, and adult. Adult tissue is most suitable:

1. a single pancreas contains sufficient tissue to reverse diabetes in one recipient; and
2. the grafts function immediately after transplantation.

There are three types of preparation of islets from adult pancreases:

1. Purified islets
2. Dispersed fragments of islet-containing tissue

3. Islet-cell isolation.

Which would be most useful for large mammals?

The critical question is: how can sufficient viable islets be harvested from a single donor pancreas? The first method yields prohibitively low quantities (< 5%). The second, proven successful by transplant studies, yields 5-10%, but the tryptic action of exocrine tissue remains. The yield with the third method has not been quantified but probably; low.

Thus, the most favorable method appears to be the use of dispersed fragments. New developments, using this approach as their basis, may further increase islet yields: venous disruption of pancreases (71) collagenase perfusion via the pancreatic ducts, and cell separation by centrifugal elutriation. In animals, increased yields of purer islets are being obtained, denting the barrier of low yield and bringing closer the possibility of clinical islet transplantation, and thus warranting more experiments. Further, the new techniques that increase islet yields permit a rational attack on the other problems with islet transplantation: optimal site, allograft rejection, definition of metabolic efficiency, and storage methods. Thus, it seems possible that islet transplantation can be used to cure diabetes mellitus while the quest for methods of prevention continues.

III MATERIALS AND METHODS

Animals and Care

Thirty-one conditioned adult mongrel dogs of either sex, weighing 15 to 25 kg, were studied. They were housed in the large-animal vivarium of the Surgical-Medical Research Institute, under the care of the investigators and with advice from a veterinarian, in accordance with the criteria formulated by the Canadian Council on Animal Care. The dogs were weighed weekly, and were permitted unrestricted exercise twice-daily. Their daily (full) diet consisted of 360 g of meat (Dr. Ballard's) and 600 g of Pow R. Pac Burger Bits.

One week after determination of the blood-glucose level and intravenous glucose-tolerance testing (I.V. GTT; 0.5 g/kg body weight), total pancreatectomy was performed. Four dogs were not further treated; the abdomen was closed, and they were maintained without insulin as apancreatic controls. The other 27 dogs were (a) maintained under anesthesia while the pancreas was processed as a graft, which was then auto-implanted immediately, or (b) the surgical procedure was completed, the pancreas was processed, and the suspension was cryopreserved until implantation later.

Postoperatively, blood glucose (BG, mg/dl) was measured daily for 4 days and twice-weekly thereafter. On day 1 (day of operation), hydration was maintained with 500 ml Ringer's lactate solution given s.c.; on day 2, this was repeated and water was allowed. On day 3, a meat diet was offered; and on day 4, the full diet. Each meal was supplemented with 8 to 10 capsules of Cotazym (Organon, Montreal). A

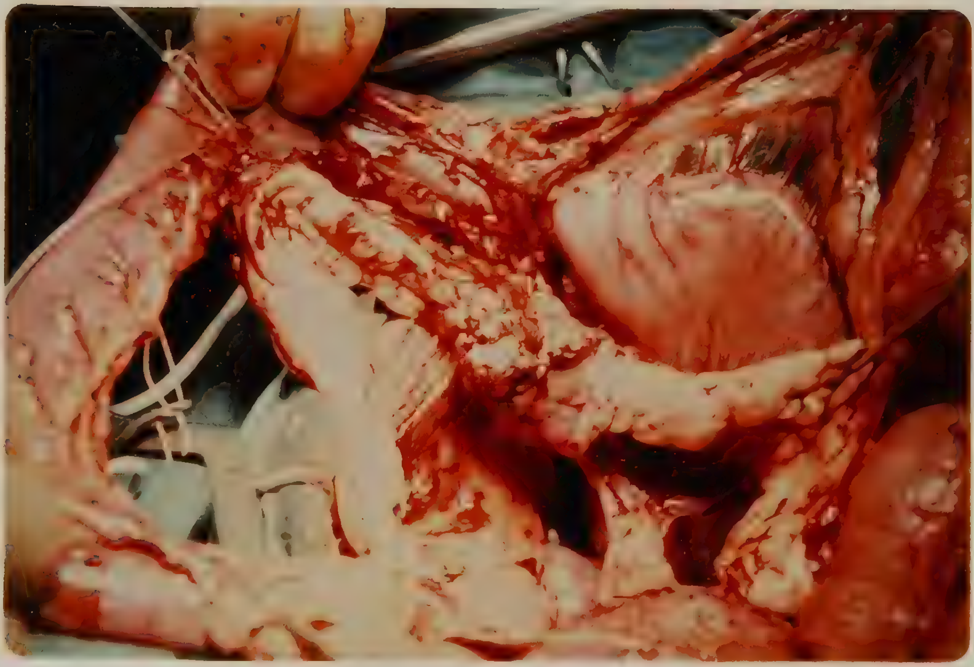
solution of penicillin with streptomycin, 2 to 3 ml, was injected i.m. during surgery and immediately postoperatively.

At the end of the study of each dog, the animal was killed with an overdose of pentobarbital sodium. Necropsy was performed, and the spleen was removed for histologic examination.

Pancreatectomy

The dogs were anesthetized with pentobarbital sodium (30 mg/kg) supplemented with Atravet (Ayerst), 0.5 ml, then intubated, and i.v. administration of Ringer's lactate at 150 ml/hr was started. Through an upper midline incision, the entire pancreas was mobilized, with all major vascular connections preserved. Both branches of the pancreatic duct were cannulated (20-22 gauge) in situ (in some dogs, only the left duct was large enough) (plate 1). A 0.2-g sample of pancreas was excised for insulin and amylase assay, care being taken to ligate the pedicle from which it was removed. Finally, the blood vessels were clamped and ligated, and the gland was removed. Immediately, the pancreas was weighed and the ducts were distended by the injection of iced Hanks' balanced salt solution (HBSS), 60 ml into the left limb and 15-20 ml into the right. The tissue was immersed in HBSS at 4°C and transferred to the laboratory for graft preparation. Meanwhile, an assistant maintained the dog under general anesthesia (in case of implantation of fresh graft) or completed the surgical procedure and instituted usual postoperative care (if the graft was to be cryopreserved).

a.



b.

Plate 1 Pancreatectomy a. Pancreas is completely mobilized, preserving vascular arcades. b. both branches of the main duct are cannulated (20-gauge) <u>in situ</u>.

Graft Preparation

A modification of the method described by Horaguchi and Merrell (28) was used (Fig. 1).

During the digestive phase, the cannulas within the ducts were connected to a perfusion apparatus and flushed with iced HBSS for 12 min at a flow rate of 10-20 ml/min (Plates 2,3). Then the perfusate was changed to a solution of 0.4% collagenase (Sigma Chemicals; type V, 250 U per mg) in HBSS at 37°C. With the method used, the digestate flows in retrograde direction through ducts of decreasing size, distends the acini, and ruptures tissue within the pancreatic lobules. The gland is never immersed in the digestate; it is suspended above the reservoir of collagenase, isolated by a 60-μ stainless-steel mesh. The digestate is then re-perfused from the reservoir. Perfusion time averages 35 min, by which time the gland is mucoid or mushy.

When digestion was judged complete, the tissue was transferred to a mincing jar containing 50 ml of enzyme stop solution {4°C HBSS ± 2% trasylol (Miles Pharmaceuticals) and penicillin-streptomycin 2.5 x 10^4 units/5 dl} . This solution was used in all subsequent steps, and is the one referred to unless otherwise specified.

In the second step, the tissue was minced for 90 sec in a mechanical mincer; it was critical not to exceed this time. The supernatant fluid was taken off and discarded, and the tissue was resuspended in solution and transferred to an Ehrlenmeyer flask.

During step 3, the tissue was dissociated by shaking the Ehrlenmeyer flask vigorously in an Evapo-Mix for 10 min at 4°C, and then filtered

through a 400 μ stainless-steel filter. This was done three times, residual unfiltered tissue being returned to the Ehrlenmeyer flask for re-dissociation and re-filtering.

Finally, the preparation was washed three times: the filtrate was centrifuged at 2000 rpm, the supernatant fluid was aspirated, and the pellet was resuspended, x 3. A sample of the final pellet, which consisted of fragments < 400 μ in diameter, was taken for insulin and amylase analysis.

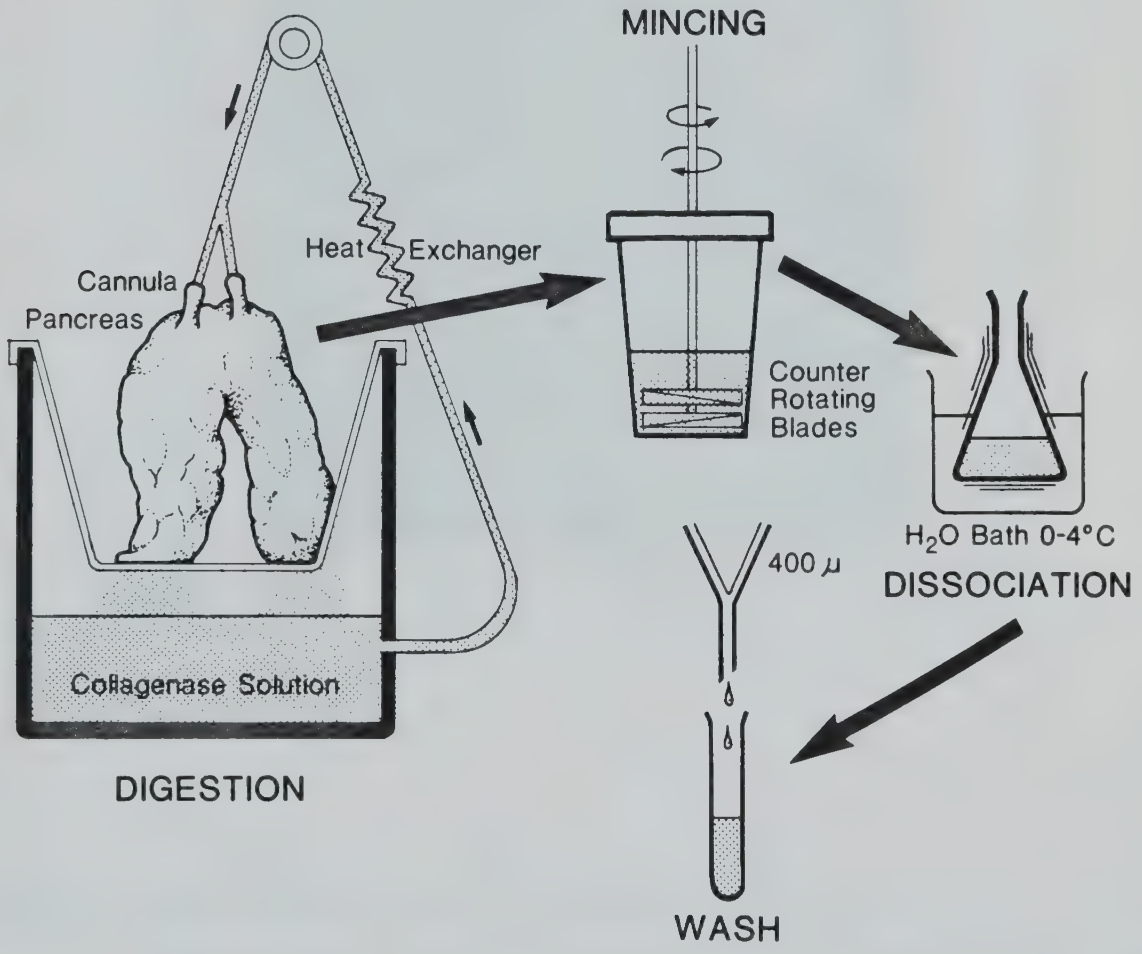


Fig. 1 Graft Preparation

Pancreases were cannulated via the ducts and were processed through 4 phases: digestion, mincing, dissociation, and washing, to prepare a suspension of islet-containing fragments.

Plate 2 Perfusion Technique. Photograph of apparatus for perfusing pancreas. The pancreas within the funnel is suspended above the reservoir of digestate; it is never immersed in the collagenase. The apparatus is set up in a laminar flow hood to preserve sterility during preparation.

Plate 3 Distended Pancreas. Canine pancreas, distended with HBSS ($4^{\circ}C$), ready for perfusion with 0.4% collagenase.

It took approximately 2 hr to prepare the suspension, which was auto-implanted in various experiments. Fig. 2 summarizes these experiments, totalling 27 auto-grafts in three groups of dogs.

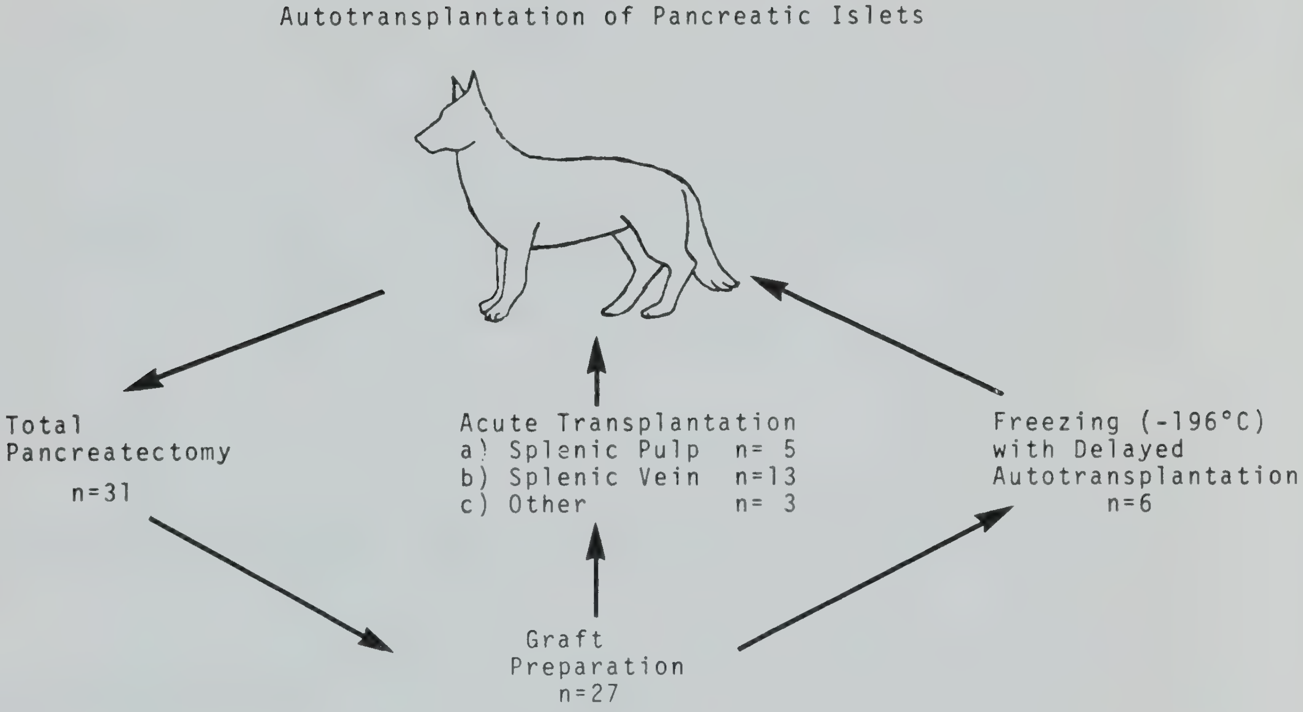


Fig. 2 Summary of experience with pancreatic islet autotransplantation, totalling 27 canine grafts. Three groups of dogs were studied: implantation by reflux into splenic veins versus injection into the splenic pulp; metabolic efficiency after splenic venous implantation; cryopreservation and thawing of the graft before implantation.

Group 1: Site and method of implantation (21 dogs)

The pellet was re-suspended in 30 ml of iced HBSS with 2% trasylol and penicillin (100 U/ml) with streptomycin (100 μg/ml). (Plate 4)

In 13 dogs, the graft was implanted via 22-gauge cannulas by reflux into superior and inferior terminal polar splenic veins (SV); (see Plate 5).

In 5 dogs, two sites in the splenic capsule (at the superior and inferior poles) were punctured with a 22-gauge needle 5 cm long; the needle was advanced 3 times into the splenic pulp (SP) at each site, the suspension being injected during withdrawal; manual pressure was applied, and the injection sites were observed for hemostasis for 10 min.

In these 18 dogs, during injection the splenic pedicle was controlled with vascular clamps, which were released 5 min after implantation.

In 2 dogs, the suspension was embolized to the stomach via 22-gauge cannulas in short gastric arteries.

In 1 dog the suspension was embolized over 30 min into the portal vein; portal pressure was monitored before, during, and after injection.

Plate 4 Graft Suspension. Suspension of islet-containing pancreas fragments, (about 9 ml) ready for implantation.

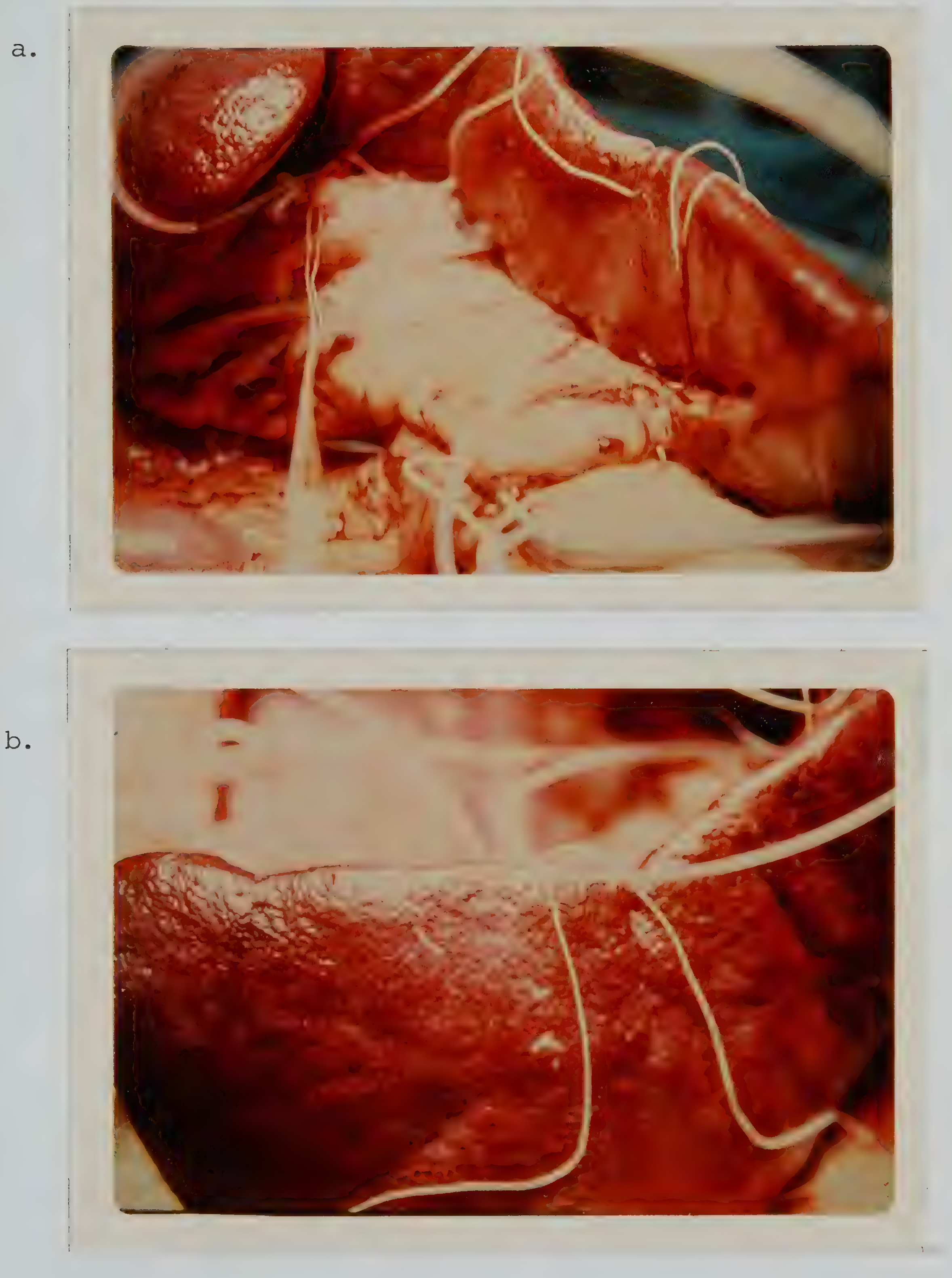


Plate 5 Splenic venous reflux of islet-tissue. a. The cannulated splenic vessels; note the vascular clamps. b. The surface of the spleen after injection; yellow strands of implanted subcapsular islet-tissue are visible.

Group 2: Metabolic efficiency of grafts (8 dogs); (See Fig. 3)

The autograft had been implanted by reflux into splenic veins. At 1 and 2-3 months post-implant i.v. GTT (8 and 7 dogs, respectively) was performed. Saphenous veins were cannulated, glucose was injected, and blood was collected at 0, 1, 5, 10, 15, 30, 60 and 90 min for assays of glucose, insulin, and glucagon. The K value (the percentage decline in glucose level per minute) was determined from samples taken at 5, 10, 15 and 30 min, with the method of least squares (72).

At 3-5 mo, anesthesia was induced for studies of insulin and glucagon secretion in central and peripheral veins, in six transplanted dogs and two healthy control dogs (plate 6). Both femoral arteries were cannulated (14 gauge) via cutdowns on recurrent femoral branches. One cannula was used for monitoring arterial pressure, and the other for blood sampling (A). The left femoral vein (V) was cannulated. Through upper midline abdominal incisions, the splenic (S) and portal (PV) veins were cannulated (18 gauge) via gastroduodenal and short gastric branches. The PV cannula tips were placed just proximal to the portal-vein bifurcation, and the S cannula tips were placed in the main splenic vein.

The left hepatic vein (HV) was entered via a right internal jugular cutdown and advancement through the right ventricle to the subdiaphragmatic inferior vena cava. The catheter was wedged, then withdrawn 1-2 cm. A 16-gauge Foley catheter was placed in the bladder and was secured with a purse-string suture. A rectal temperature probe was inserted, and body temperature was maintained at 37°C with a warming blanket. Flow probes (size 8-10 French) were placed on the hepatic

artery after the gastroduodenal branch had been ligated, and on the portal vein (size 16 or 17). After calibration of a Beckman recorder, flows were monitored. Baseline BG, hematocrit (Hct), and blood gases were determined. Finally, a loading dose of heparin (100 U/kg) was administered.

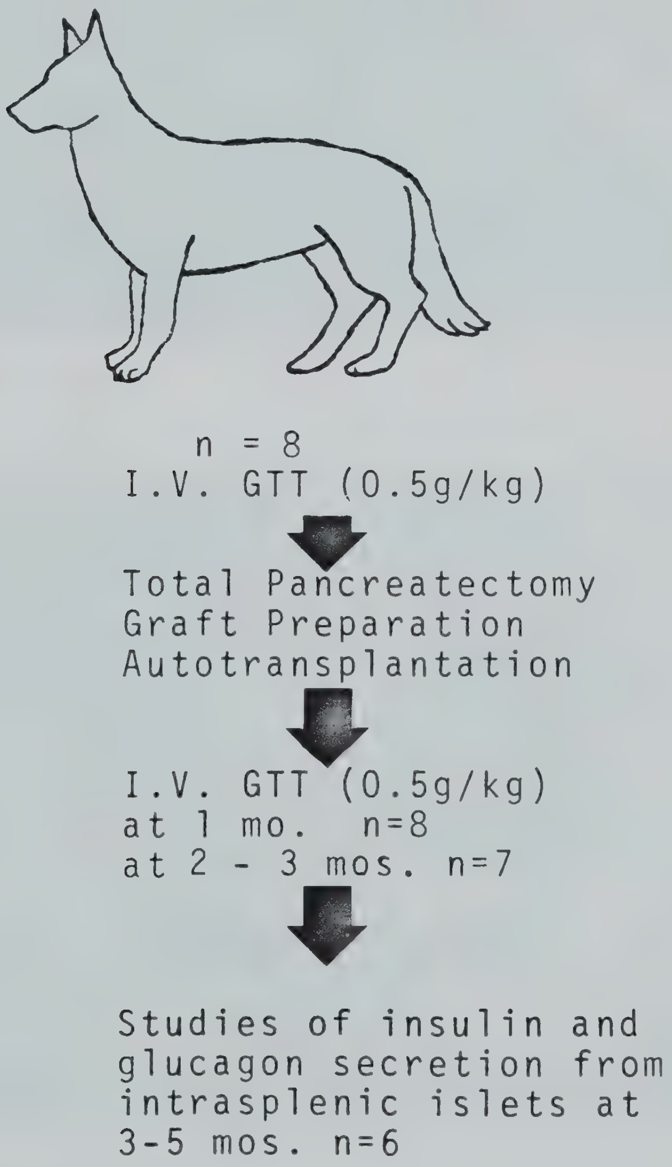


Fig. 3 Schema for metabolic studies of pancreatic islets implanted by reflux via splenic veins.

a.

b.

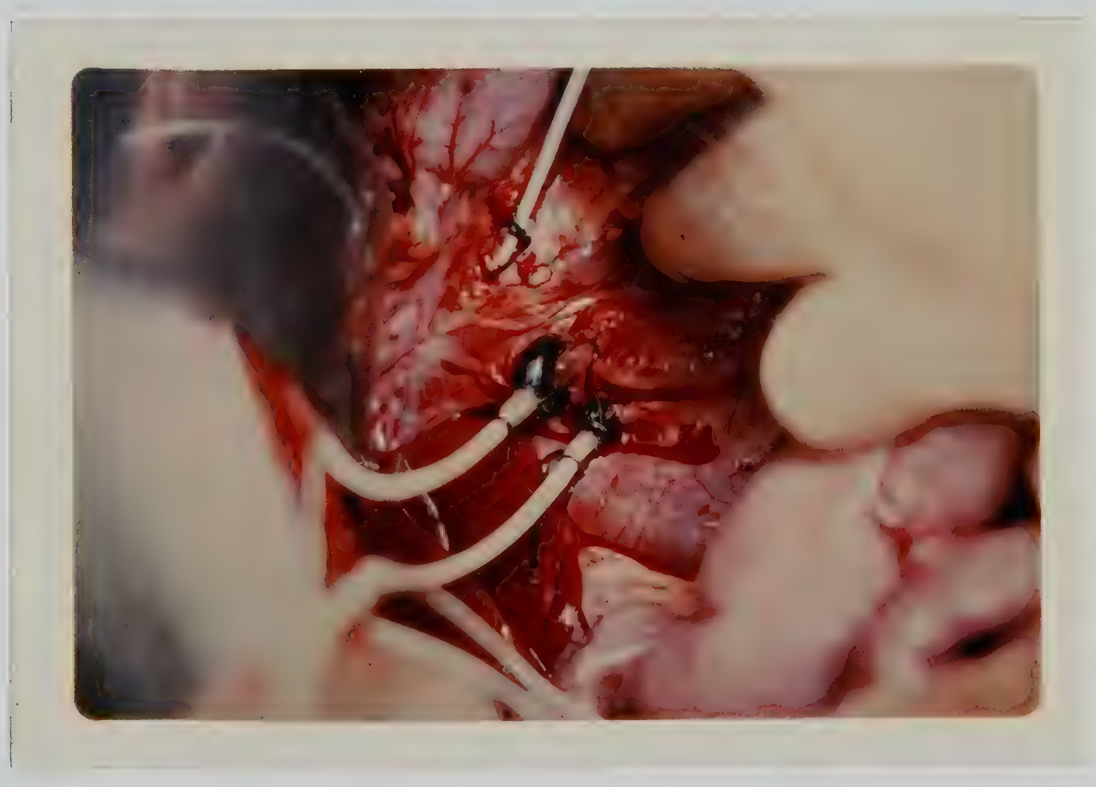


Plate 6 Cannulation studies for islet function. a. All cannulas prepared for sampling. b. Surgeon's view of placement of electromagnetic flow probes on portal vein and hepatic artery. The cannula enters the portal vein, just below the common bile duct.

During cannulation studies BG was determined and insulin and glucagon were assayed during five periods of study (Fig. 4). Samples were drawn at 1, 5, 10, 20 and 40 min in each period; in period 2, additional samples were drawn at 60 and 70 min. Systemic blood pressure, portal and hepatic artery flows, urine output, Hct, and blood gases were monitored throughout, and i.v. flows were adjusted accordingly to maintain normovolemia. The recorder was re-calibrated during each period. At the conlusions of the experiments, the animals were killed with an overdose of sodium pentobarbital while still anesthetized. Spleens were examined grossly, and samples from the superior and inferior poles were preserved for histology. After death, the site of the cannula tips was confirmed by direct vision and a mechanical zero was determined for the flow probes. The K value for decline in glucose concentration was determined from the values 5, 10, 20 and 40 min after the second challenge with glucose.

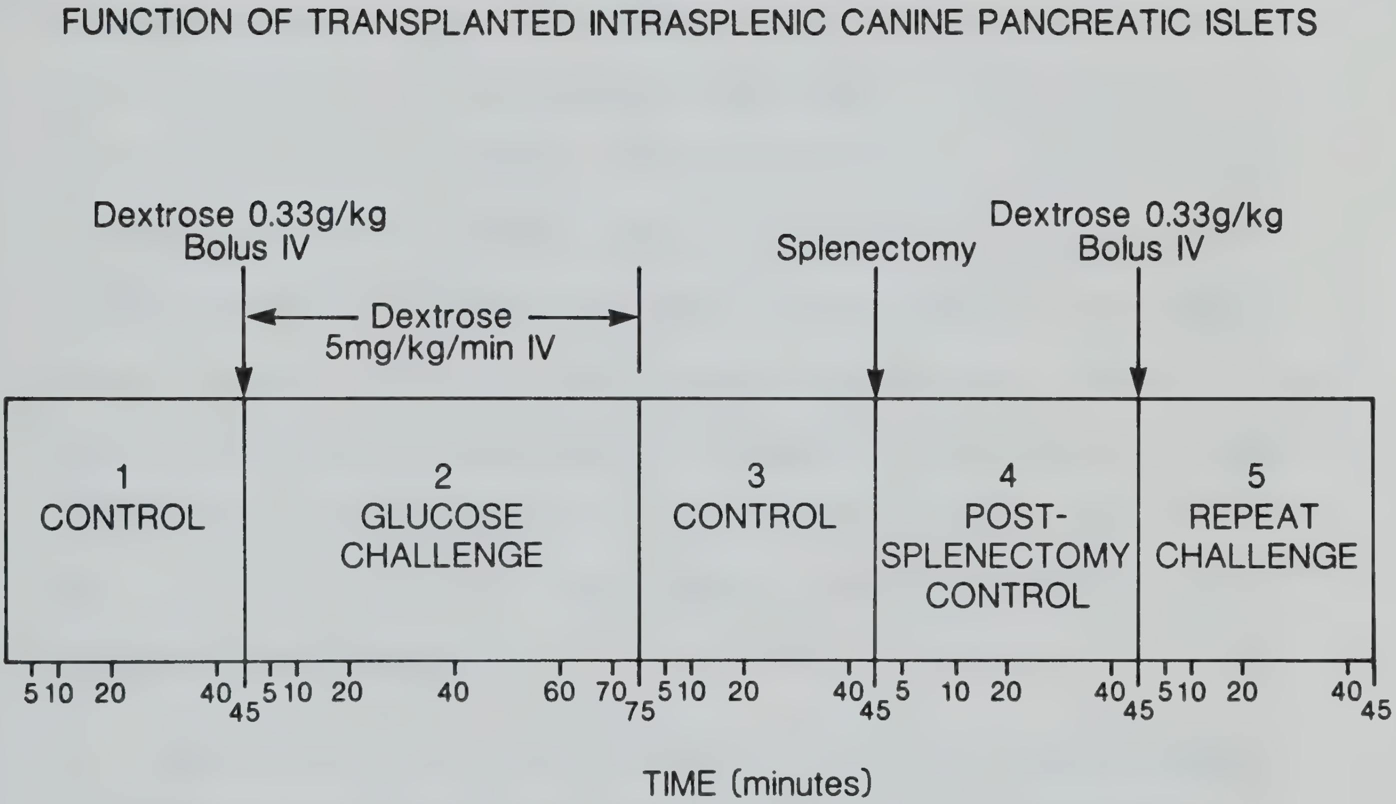


Fig. 4 Test periods for insulin and glucagon secretion from islets implanted into splenic veins, 3-5 mo after transplantation. Studies were made during 5 periods (see text).

Group 3: Graft storage by cryopreservation

After total pancreatectomy the abdomen was closed. The graft was prepared, and the final pellet was resuspended with an equal volume of medium 199, 10% fetal-calf serum, and 2% trasylol. This suspension was frozen, using a method devised by Rajotte et al for rat islets (31). Briefly, the tissue is suspended in stepwise increasing concentrations of the cryoprotectant dimethylsulfoxide (Me_2SO), as shown in Fig. 5. Exposure to 2M Me_2SO was at 0°C in 5 dogs and 25°C in 2. The suspension was placed in test tubes (4 ml, containing 0.5 ml of tissue). Two smaller tubes were designated for perfusion and insulin/amylase assay, all were frozen. They were supercooled to -7.5°C, then nucleated with ice crystals while being shaken manually to ensure uniform suspension throughout the ice. Fifteen min was permitted to release the latent heat of fusion then the frozen samples were placed in freezing Dewars for controlled cooling (0.25C°/min) to -75°C. These were then plunged into liquid nitrogen and stored at -196°C for 48 hr.

Meanwhile, the dogs were maintained with fluids subcutaneously and 6-8 U of NPH insulin was injected s.c. on day 1. On day 2, the graft was thawed at room temperature (∿ 45 min). The tubes were centrifuged at 2000 rpm, the 2M Me_2SO was aspirated, and 2.5 ml of 0.75M sucrose was added to each aliquot. The preparations were allowed to equilibrate for 30 min. The sucrose content was diluted in 4 steps of 5 min each, by the addition of medium 199 with 10% fetal-calf serum, 2% trasylol, and penicillin 100 U/ml with streptomycin 100 μg/ml. The mixture was centrifuged and supernatant fluid was aspirated. The

pellet was prepared for implantation: 0.1 ml was taken for insulin/amylase assay and the remainder was resuspended in 30 ml of iced HBSS with 2% trasylol and penicillin-streptomycin. The dogs were anesthetized and their incisions re-opened, and the tissue was transplanted by reflux into splenic veins.

Fig. 6 summarizes the thawing procedures.

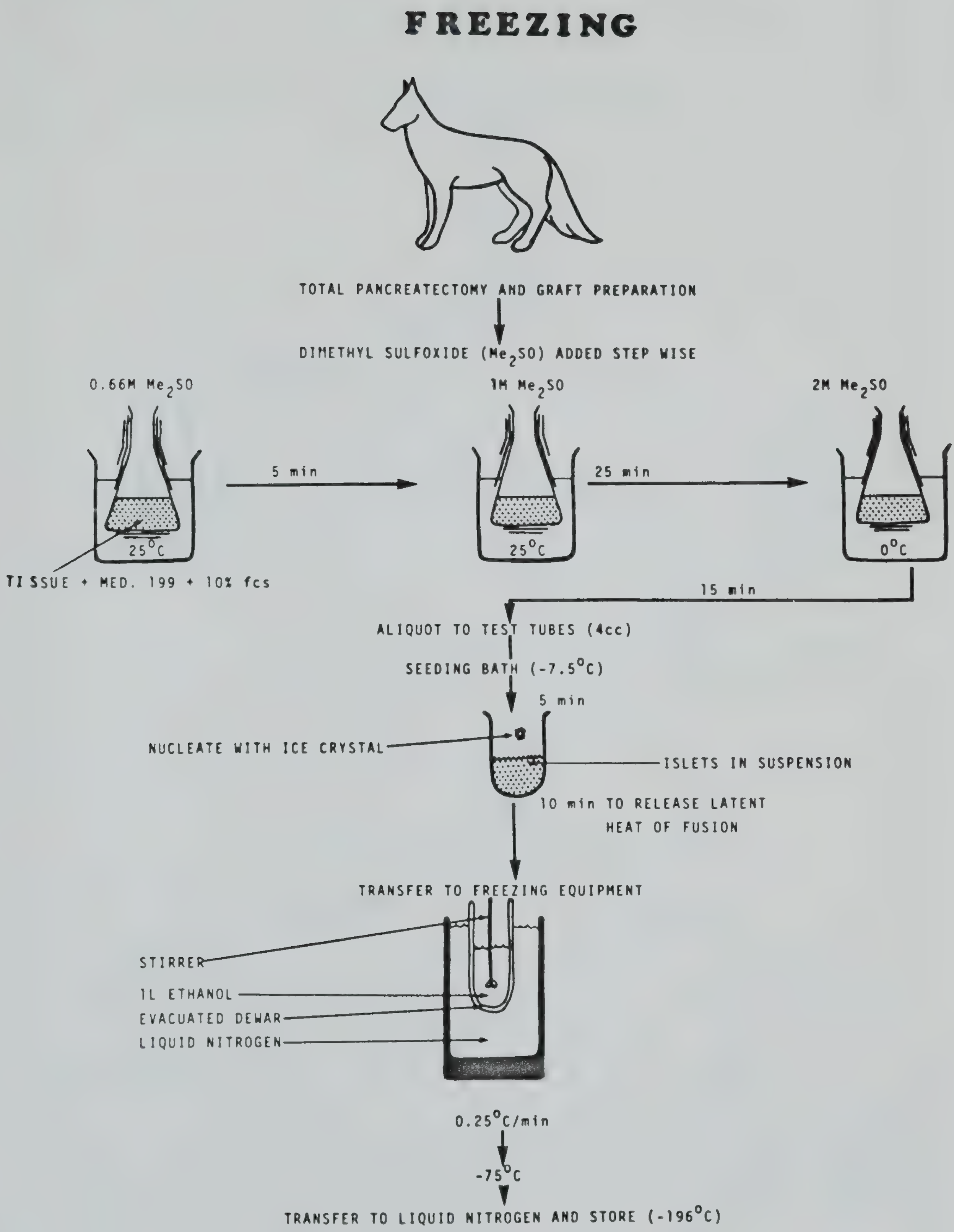


Fig. 5 Freezing of islet-containing tissue.

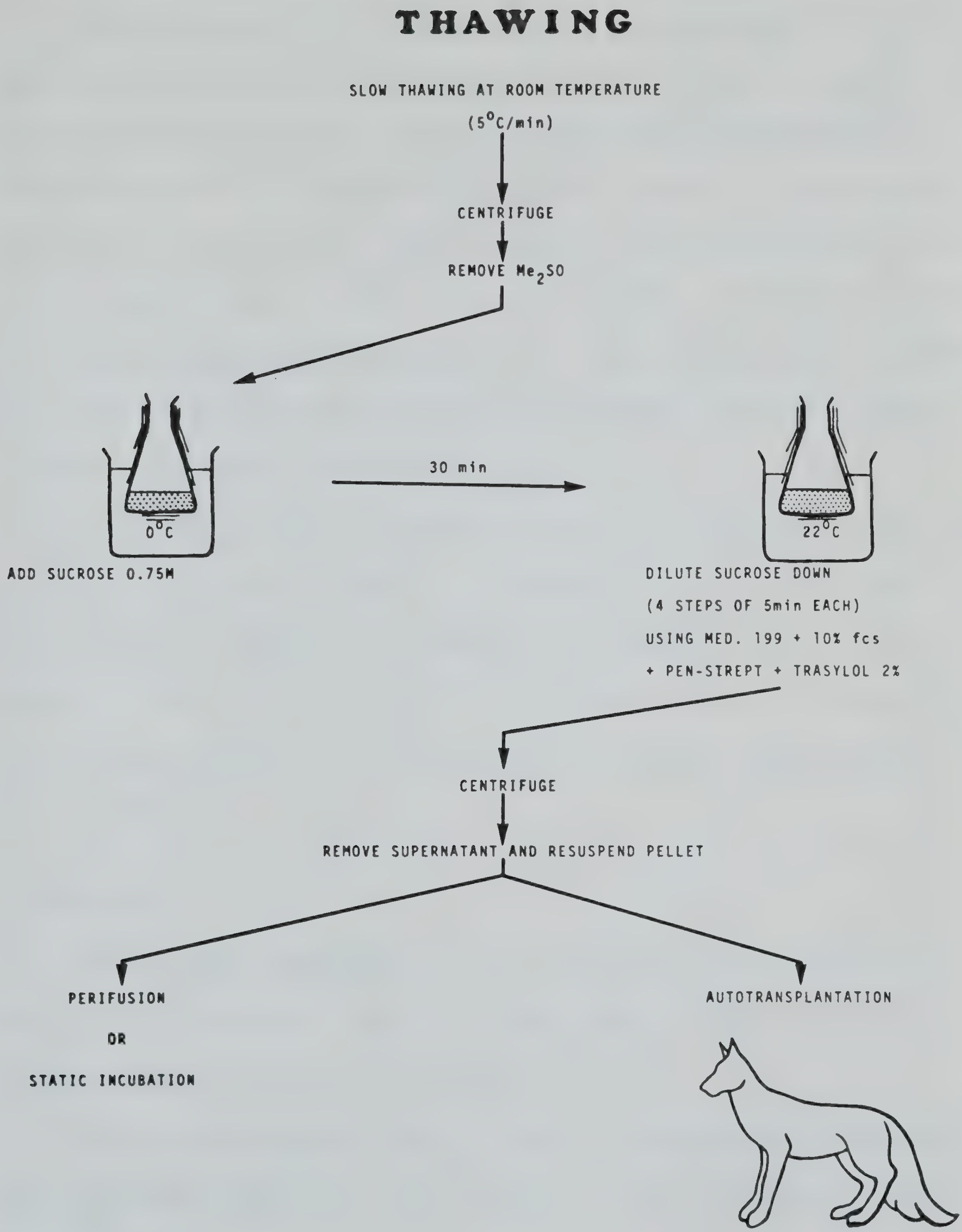


Fig. 6 Thawing of islet-containing tissue.

Tissue Analysis

Fresh pancreas (0.2 g) and aliquots of the suspension (0.1-0.2 ml) were weighed, homogenized, and sonicated at 50 watt-sec for 30 sec for insulin and amylase assays. Insulin (9 dogs) was extracted after sonication in 5 ml of ice-cold acid:alcohol. At 24 hr, the aliquots were neutralized, diluted x 100 in Delbecco's phosphate buffer solution (Gibco), then frozen at $-20^{\circ}C$ until insulin assay. Insulin was measured by double-antibody radioimmunoassay (73), using porcine insulin standards. Amylase (11 dogs) was determined by the amylochrome technique (Roche Diagnostics) in 10-ml aliquots of saline.

It was assumed that the insulin and amylase contend was proportional to B-cell and exocrine content of pancreases and grafts (60). The graft's insulin content was expressed as a percentage of the organ's insulin content, to indicate the B-cell yield. The graft's amylase content was expressed in a similar manner to estimate the exocrine tissue remaining.

Plasma Assays

Blood glucose was measured by the hexokinase method, using an IL Multistat-III analyzer. During the cannulation studies, the glucose-oxidase method was used with a Beckman analyzer.

Plasma immunoreactive insulin (IRI) was determined by double-antibody radioimmunoassay (73), using porcine insulin standards (Wellcome Reagents Ltd).

Plasma immumoreactive glucagon, (IRG) was determined by radioimmunoassay, using a single-antibody precipitation method (74) with

porcine standards for crystalline glucagon (Eli Lilly).

All blood samples were collected into tubes containing heparin or potassium oxalate and serum was separated promptly after collection. When glucagon was assayed, 500 KIU trasylol was added to each 1 ml of whole blood and the tubes were kept at 4°C.

Histology

i) Light Microscopy

Graft tissue, fresh pancreas, and transplanted spleens were fixed in Bouin's fixative or 10% formalin. Gomori's aldehyde fuchsin with Mallory's trichrome counterstain was used. Immunocytochemical location of insulin and glucagon was done with modifications of the unlabeled antibody enzyme method of Sternberger and Erlandsen et al (75,76). (DAKO PAP Kit$_{TM}$ - supplied by Cedarlane labs).

Statistical Analysis

All results were expressed as mean ± SEM. Analyses were with Student's t test for paired or unpaired data as applicable. Differences are stated as significant when $P < 0.05$ unless indicated otherwise.

IV RESULTS

Graft Preparation

The characteristics of grafts of islet-containing tissue are summarized in Table 1.

Mean graft volumes were 10 ml. B cell yield, as reflected by insulin recovery in the graft, was 24%. The exocrine tissue remaining in grafts, was reflected by a 25% amylase content. Histologic studies revealed islets and islet-fragments 50-100 μ diameter, with some exocrine contamination.

TABLE I

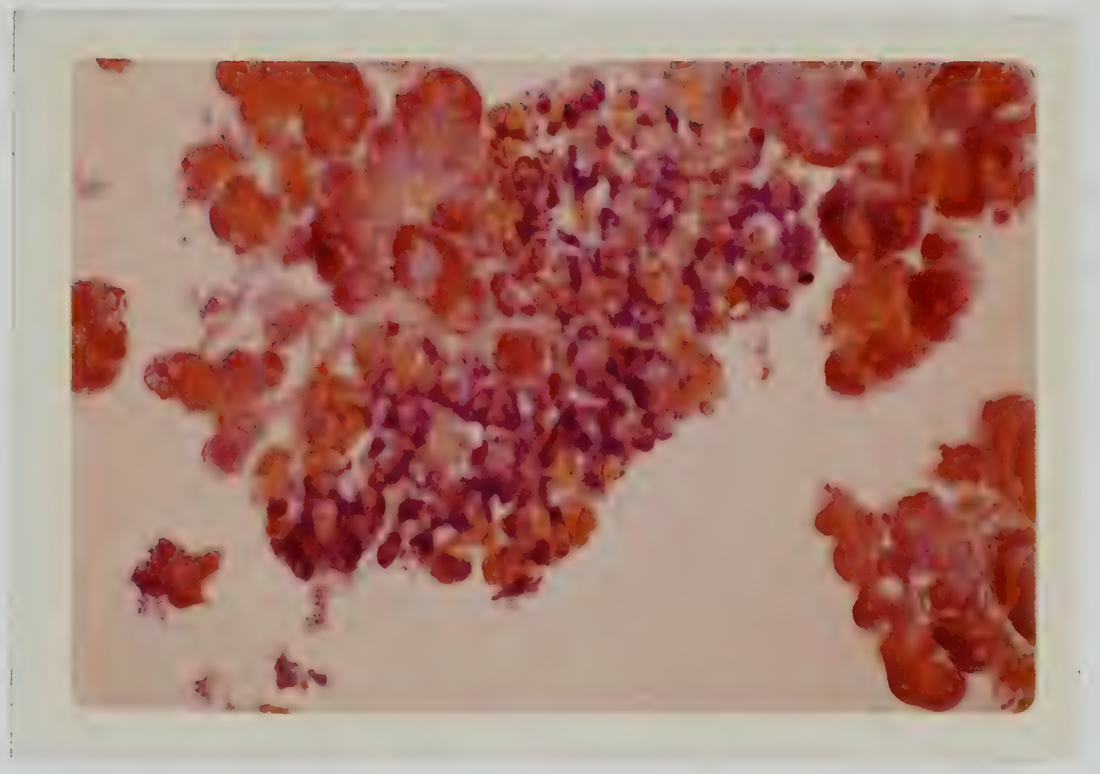
Total Insulin* and Amylase* Content of Pancreases and Grafts

	Weight (g)	Total tissue insulin content (mU)	Percentage recovered (N = 9)	Total tissue amylase content (x 10^3 du)	Percentage remaining (N = 11)
Pancreas	44 ± 4	13,577 ± 3340	100	75 ± 13.57	100
Graft	10 ± 1	3,311 ± 1029	24	19 ± 4.23	25

* Mean ± SEM

± Spun tissue volume (ml)

a.



b.

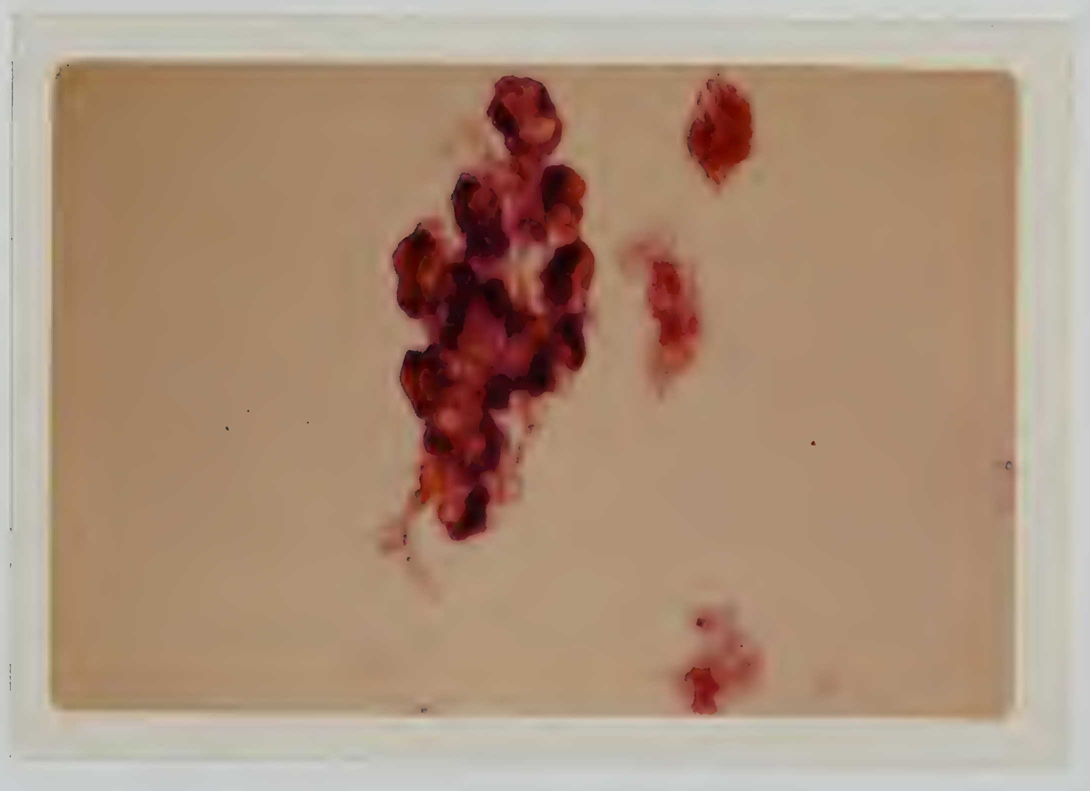


Plate 7 Histology of grafts. Harvested islets, stained with Gomori's Aldehyde fuchsin. a. islet with acinar contamination (x 40). b. isolated islet (x 100).

Site and method of transplantation (Fig. 7)

The apancreatic controls (4 dogs) became severely diabetic and survived for 10 ± 3 days, their mean BG being 343 ± 11 mg/dl terminally. The mean BG concentration during the initial 10 days was always significantly higher ($p < 0.01$) in the controls than in the splenic-pulp group ($n=5$). However, in two of the latter the BG ranged between 150 and 200 mg/dl from 2 to 4 wk post-implantation and then rose into hyperglycemic range; in all five, the BG concentration rose steadily until death. By contrast, implantation by reflux via the splenic veins (13 dogs) resulted in normoglycemia for up to 5 mos (Table II). Comparison of BG values showed this group significantly different from the apancreatic controls and from the splenic pulp group ($p < 0.001$ for both). Two of the dogs in the splenic-vein group died within hours of surgery (see Discussion). In a further two the vein wall was punctured during cannulation; hyperglycemia ensued, and the dogs were killed at 2 wk. The remaining nine became normoglycemic by 5 days: 1 was killed at 2 wk (distemper) and 1 at 6 wk (became diabetic after sepsis arising in a leg ulcer (see Discussion). A third dog died of intestinal obstruction (duodenal stenosis) at 9 wk.

At 5 mo follow-up of the remaining normoglycemic dogs (Table II) the mean BG value was 91 mg/dl.

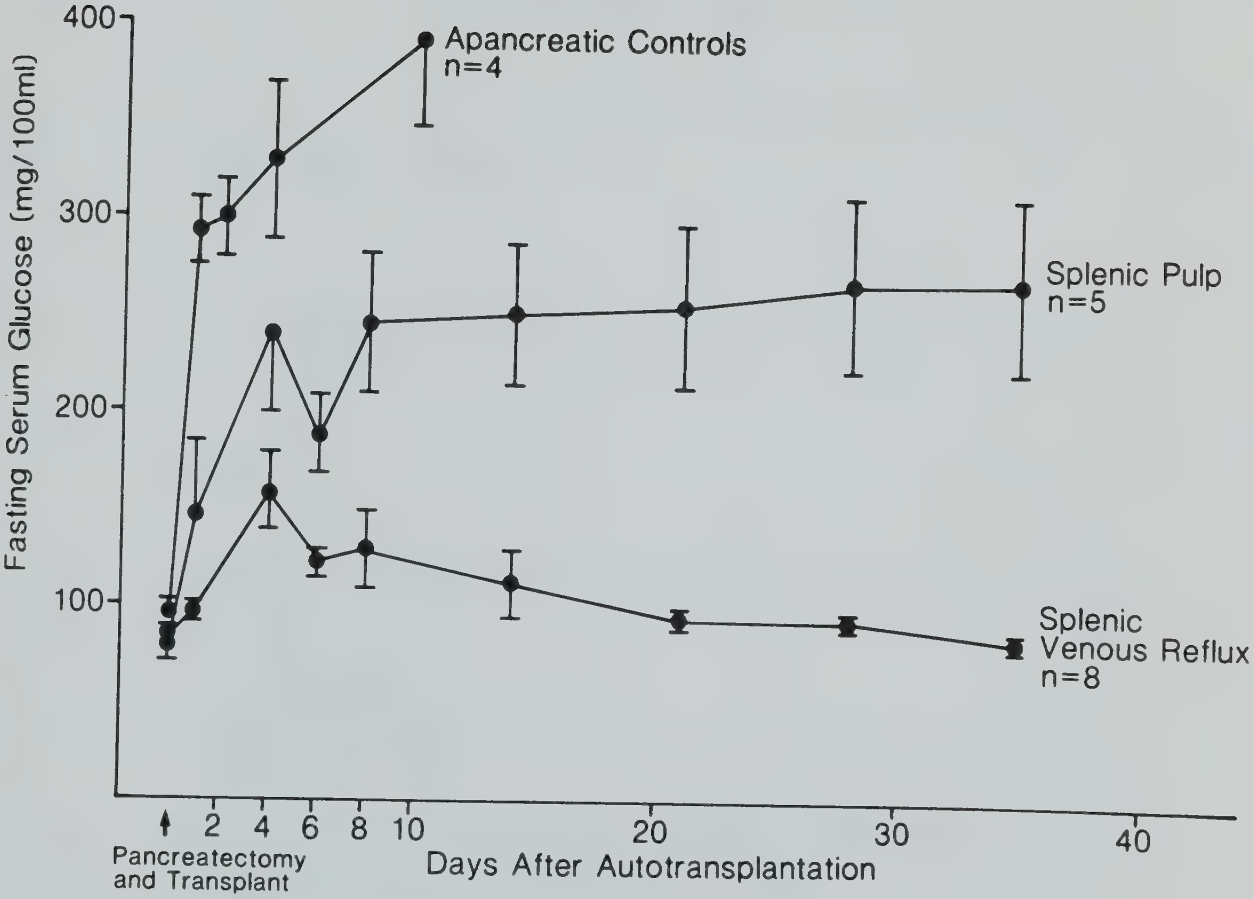


Fig. 7 Comparison of 2 methods of intrasplenic transplantation of pancreatic islets. APC dogs had significant higher b.g. than SP dogs ($p < 0.01$). SP dogs had significantly higher b.g.than SV dogs ($p < 0.001$).

TABLE II

Mean fasting BG concentration (±SEM) in dogs normoglycemic after implantation of autogenous islets via splenic veins

Time post-operatively (months)	1	2	3	4	5
n	8	7[*]	6[†]	4[‡]	4
Serum glucose (mg/dl)	94±4	97±9	88±6	85±6	91±13

[*] One dog had been killed at 6 wk because of profound sepsis and resulting hyperglycemia.

[†] One (normoglycemic) dog had died of intestinal obstruction at 9 wk

[‡] Two (normoglycemic) dogs had been killed at the conclusion of hepatic insulin-extraction studies at 3 mo.

Morphology

Grossly, spleens of the intrapulp group were fibrotic and on light microscopy, there were abundant pancreatic acini; no islets were visible. (Plate 8) In recipients of grafts refluxed into splenic veins, the spleens were supple. Histologically, islets were prominent and at 5 mo the pancreatic acini had atrophied or disappeared (Plate 9).

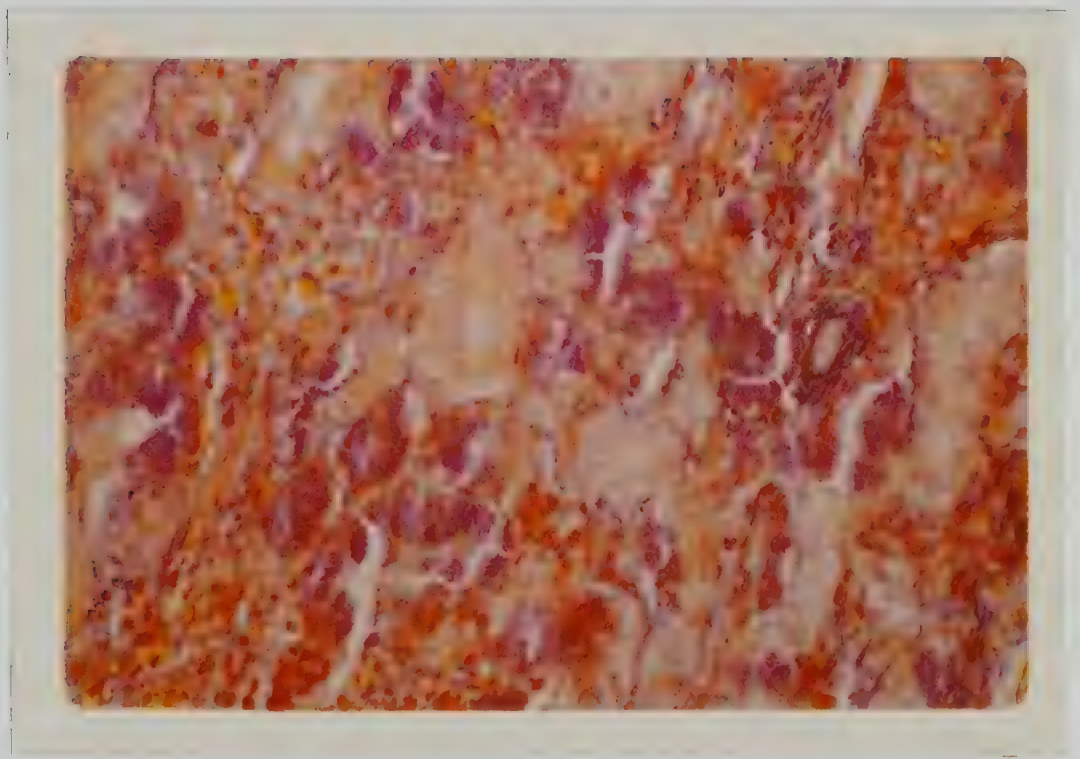


Plate 8 Section of spleen taken 6 wk after intrapulp implantation of autogenous islet tissue showing abundant pancreatic acini with prominant zymogen granules and no islets (Gomori-Aldehyde Fuchsin x 40).

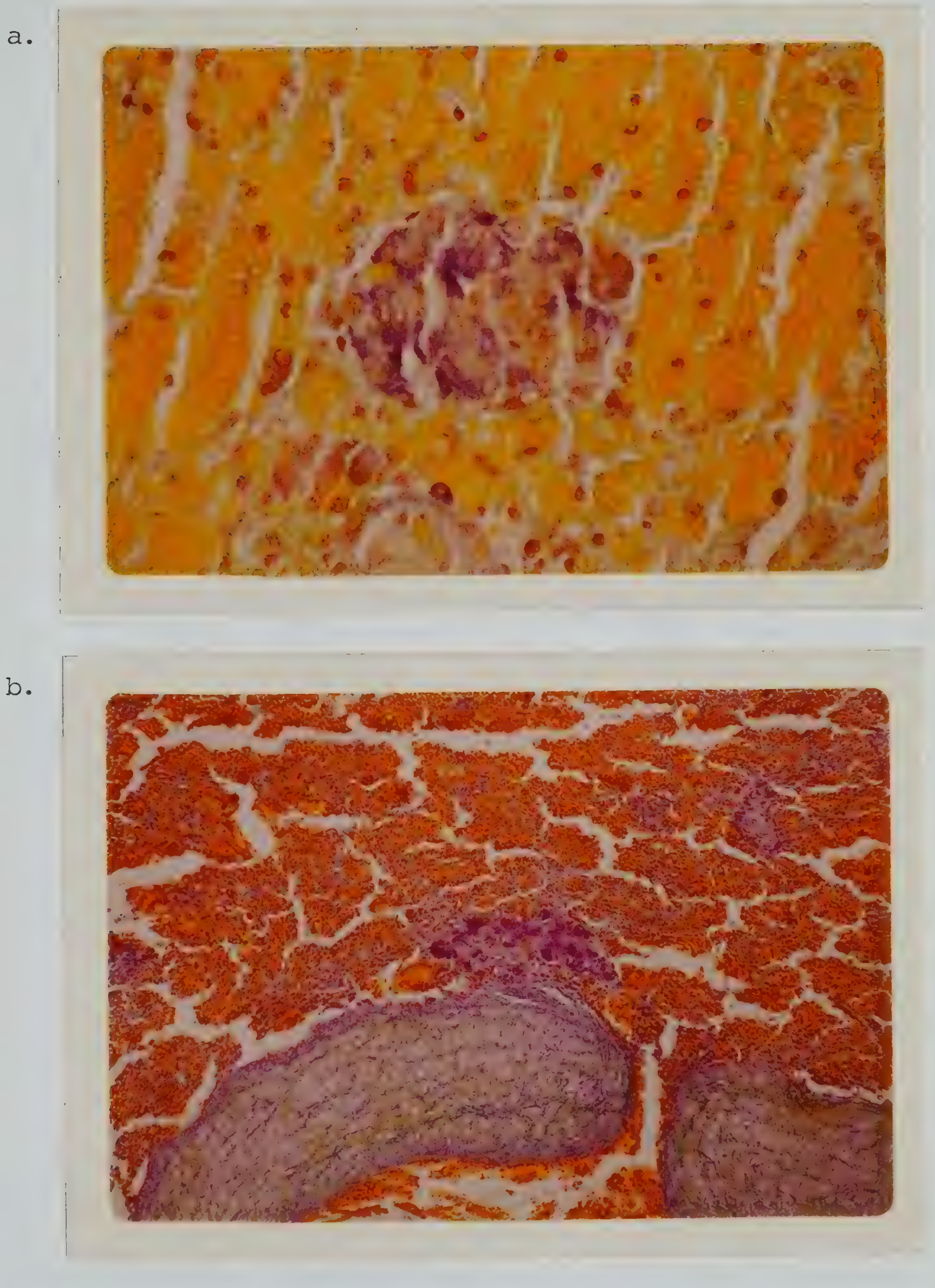


Plate 9 Spleens of recipients of pancreatic implants refluxed into splenic veins, showing islets within venous sinusoids. Staining with Gomori's aldehyde fuchsin. a. Acutely after implantation (x 100); b. 5 mo after implantation (x 40).

Metabolic Studies (Fig. 8)

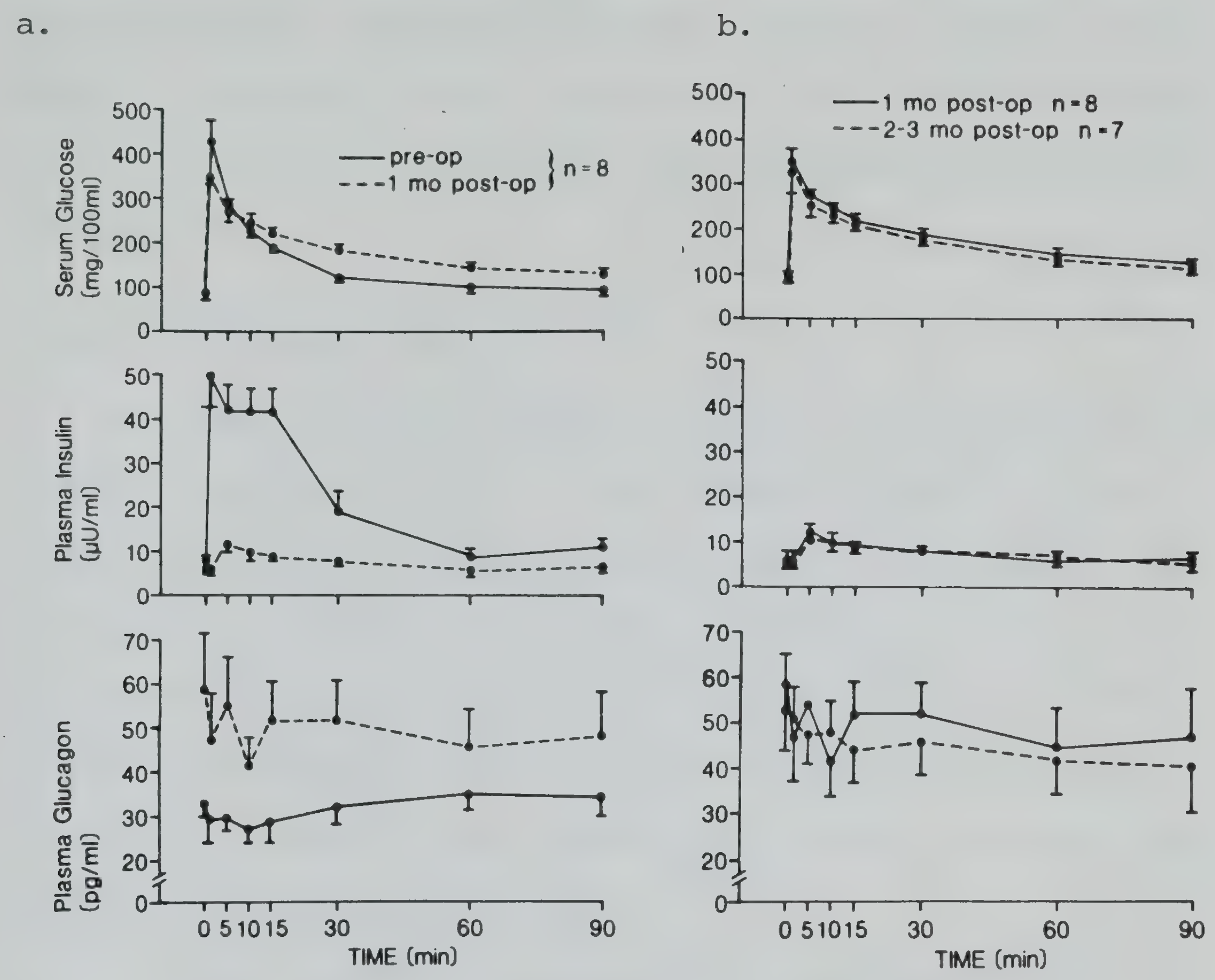


Fig. 8 Serum glucose, insulin and glucagon values (mean ± SEM) recorded during GTT at 1 mo (A) and 2-3 mo. (B) post-operatively compared with values pre-operatively. K values: 3.4 ± 0.2% preop; 1.4 ± 0.1% at 1 mo; 1.5 ± 0.1% at 2-3 mo.

In the recipients of grafts refluxed via splenic veins, comparison of the GTT results showed a slower decline in glucose concentration at 1 mo (K = 1.4 ± 0.1%) than pre-operatively (K = 3.4 ± 0.2%). The insulin peaked at 1 min (50 ± 5 µU/ml) pre-operatively but at 1 mo the peak (12 ± 1 µU/ml) was shifted to the right at 5 min. Serum-

glucagon levels showed no consistent decline during the GTT pre-or postimplantation. Comparison of the GTT results at 1 mo and 2-3 mo showed no significant change: K values are 1.5 ± 0.1%; insulin peaks at 11 ± 1 μU/ml; glucagon changes little from basal fasting levels of 53 ± 8 pg/ml.

Cannulation Studies (Figs. 9-14)

In 5 dogs who received implants by reflux via splenic veins, serum glucose in peripheral veins, and the corresponding levels of serum insulin and glucagon in splenic, portal, hepatic and peripheral veins were plotted 3-5 mo after implantation. The results are shown for 5 periods of study, as follows: initial control; a bolus of glucose followed immediately by constant infusion; second control period; splenectomy followed by third control period; bolus of glucose. Three points were especially noteworthy:

1. During the first glucose challenge, two peaks of insulin secretion were noted in splenic veins, the first (55 ± 13 μU/ml) occuring at 1 min; the second at 60 min (77 ± 15 μU/ml) (fig. 9). These peaks are not reflected in peripheral veins, and were not accompanied by a decline in glucagon levels (fig. 10).

2. At no time, during the glucose challenge, did an insulin peak occur in hepatic veins (fig. 9).

3. After splenectomy, splenic vein sampling was terminated, portal venous insulin and glucagon dropped respectively, from 22 ± 6 μU/ml and 226 ± 34 pg/ml to 3.6 ± 0.4 μU/ml and 37 ± 3 pg/ml (figs. 9,10). This was accompanied by a diabetic curve for

decline of glucose (K = 0.7 ± 0.1%) (fig. 11).

Not plotted are the results of a sixth dog who became hypovolemic during the second control period. In that dog splenic venous insulin levels, which had peaked at 76 μU/ml at 1 min and 250 μU/ml at 70 min, continued to climb to 580 μU/ml during the hypovolemic episode. This was accompanied by a rise in glucagon levels to 1236 pg/ml in the splenic venous effluent. In figures 12-14, the results after the same cannulation studies are plotted for 2 normal control dogs. After glucose challenge in period 2, portal insulin values increased to 132 ± 53 μU/ml in 1 min, and rose to peak levels of 259 ± 140 μU/ml at 40 min (fig. 12). These were higher than transplanted dogs, whose values at 1 min (21 ± 5 μU/ml) rose to peak levels of 28 ± 6 μU/ml at 70 min.

Glucagon levels in normal dogs did not decline significantly during glucose challenge (fig. 13). After the sham splenectomy, the rate of glucose decline was 1.8 ± 0.1% per minute after repeat glucose challenge (fig. 14) compared with 0.7 ± 0.1% after splenectomy in transplanted dogs.

Immunocytochemistry

Islets within spleens removed 3-5 mo. after implantation showed degranulation after immunocytochemical localization of insulin (plate 10) or glucagon (plate 11). Some islets had loss of granulation centrally on insulin staining.

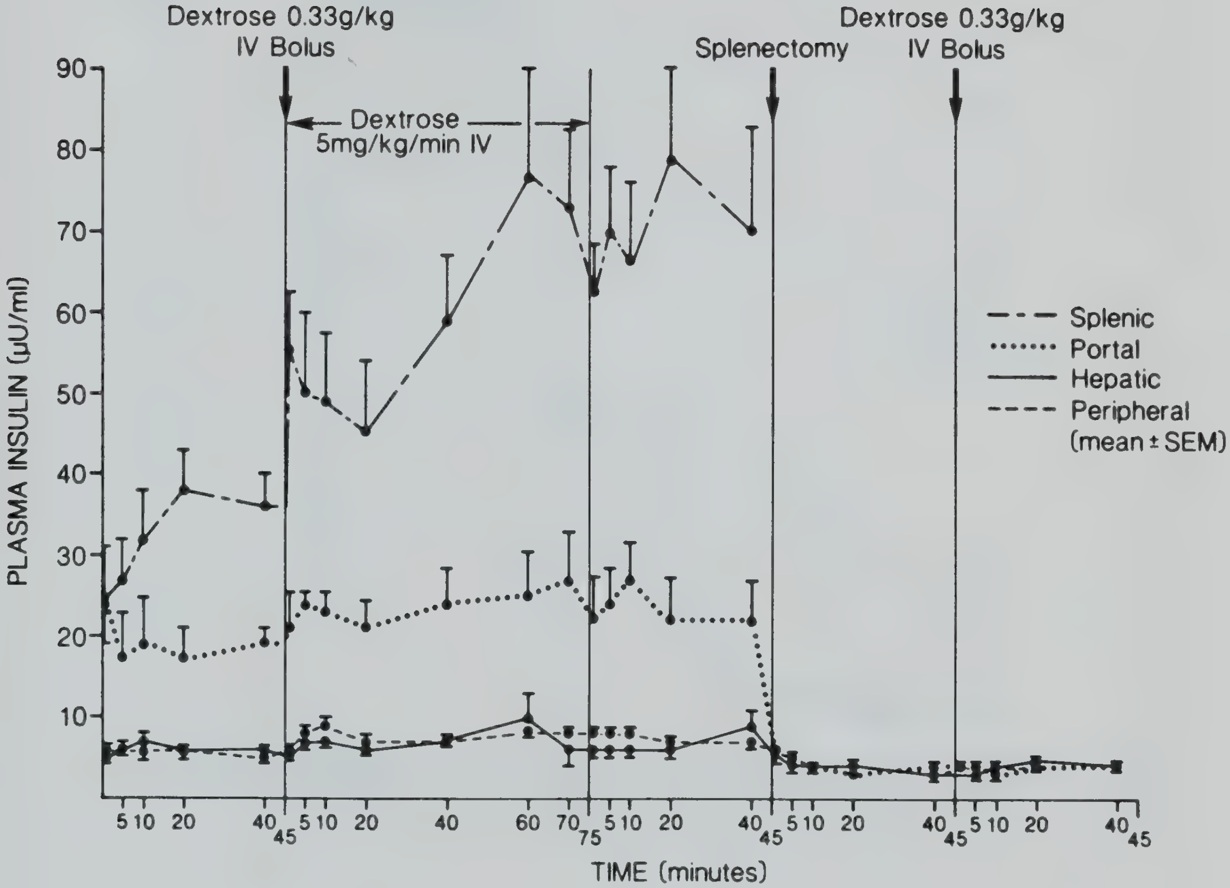


Fig. 9 Insulin secretion into splenic, portal, hepatic and peripheral veins in recipients of transplanted islets (3-5 mo after implantation).

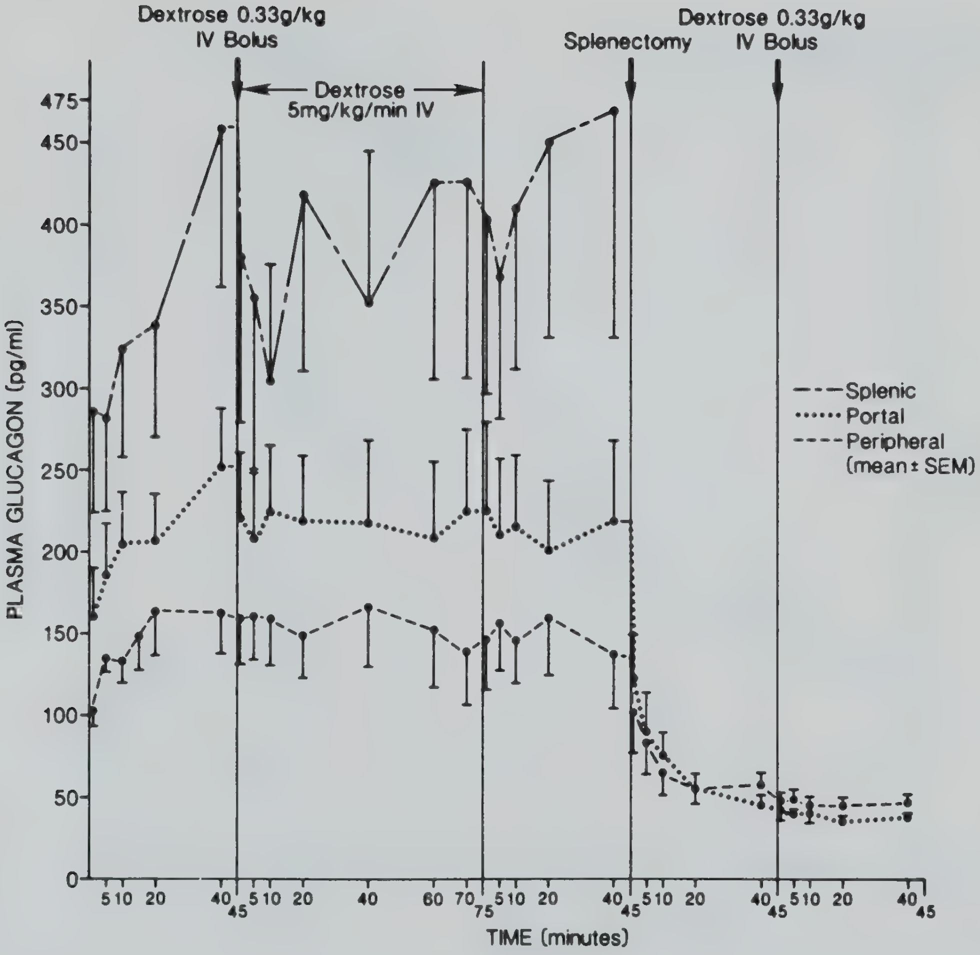


Fig. 10 Glucagon secretion into splenic, portal and peripheral veins in recipients of transplanted islets (3-5 mo after implantation).

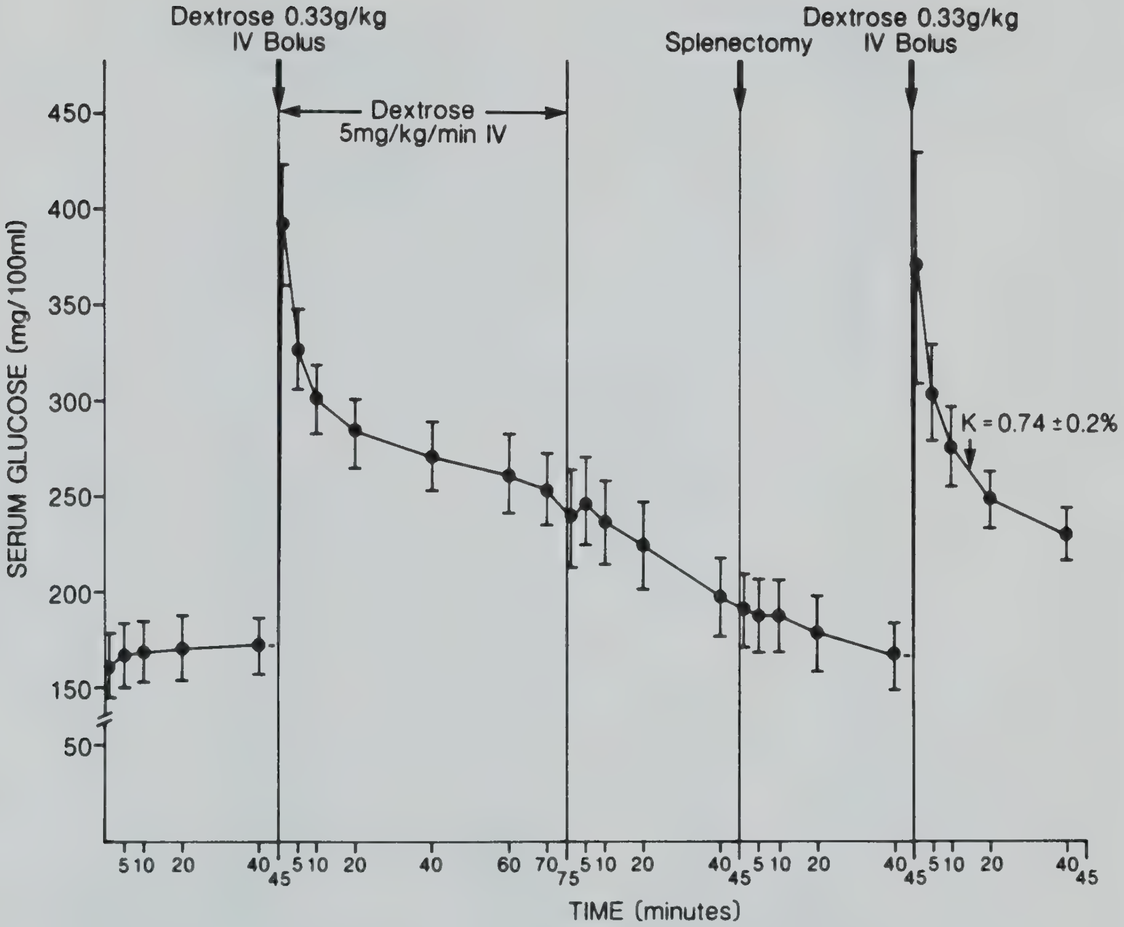


Fig. 11 Glucose levels in peripheral veins of recipients of transplanted islets during cannulation experiments. Note the diabetic decay curve (K < 1%) after repeat challenge with glucose following splenectomy (3-5 mo after implantation).

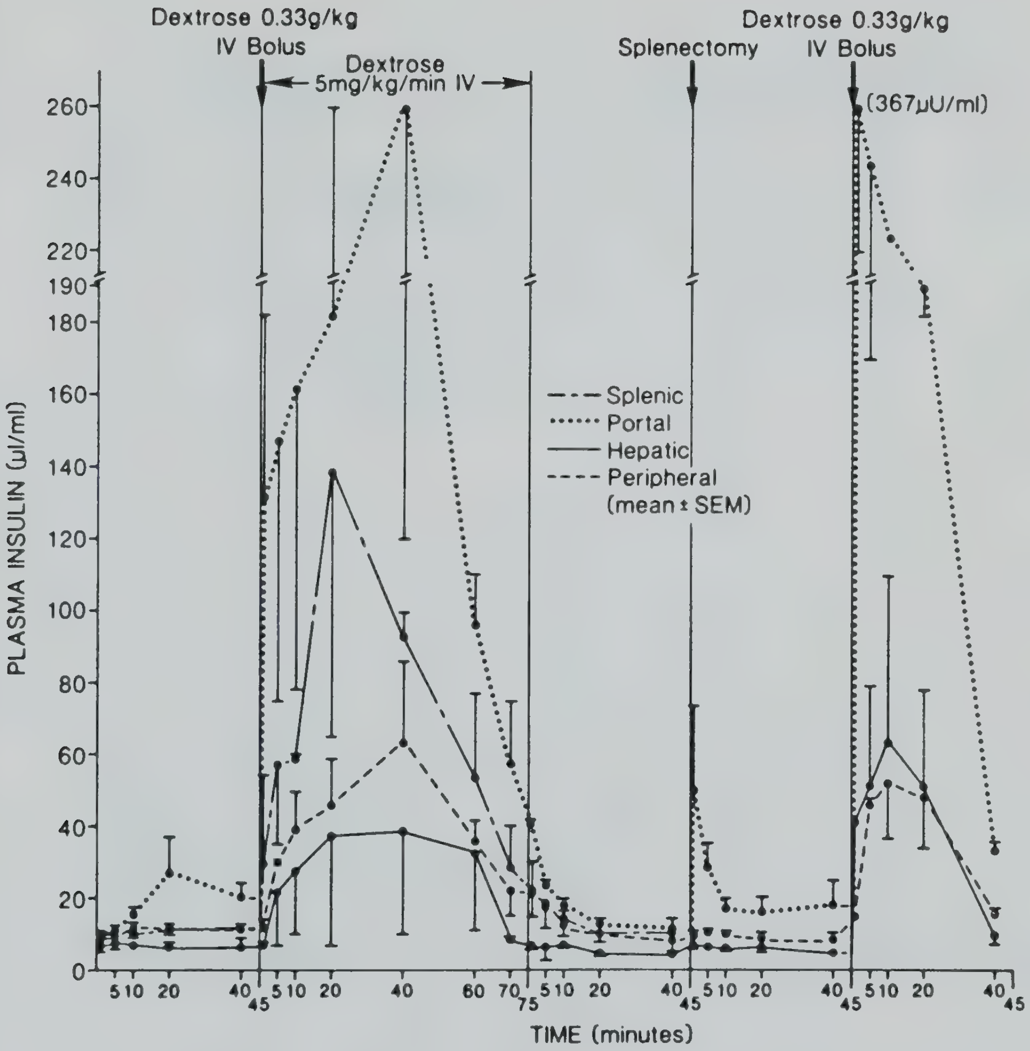


Fig. 12 Insulin secretion in splenic, portal, hepatic and peripheral veins of normal control dogs after glucose challenge.

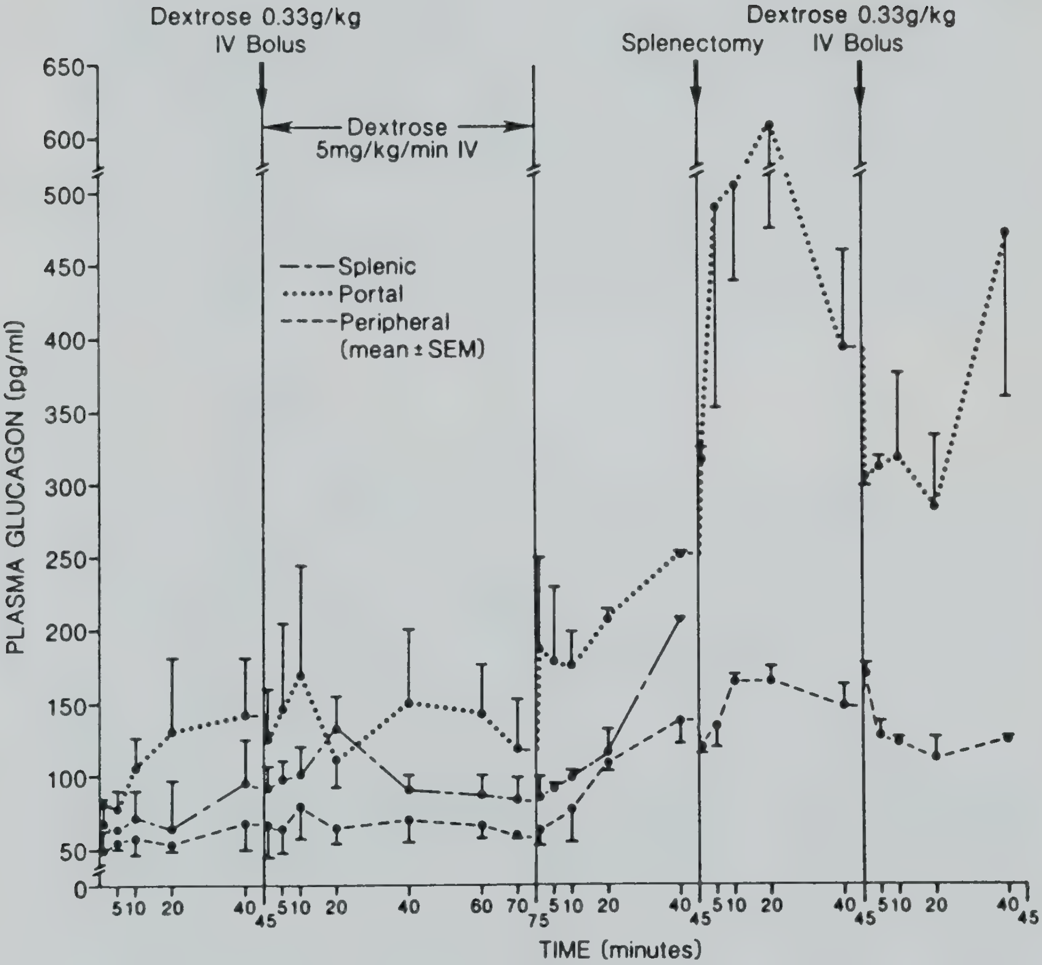


Fig. 13 Glucagon secretion in splenic, portal and peripheral veins of normal control dogs after glucose challenge.

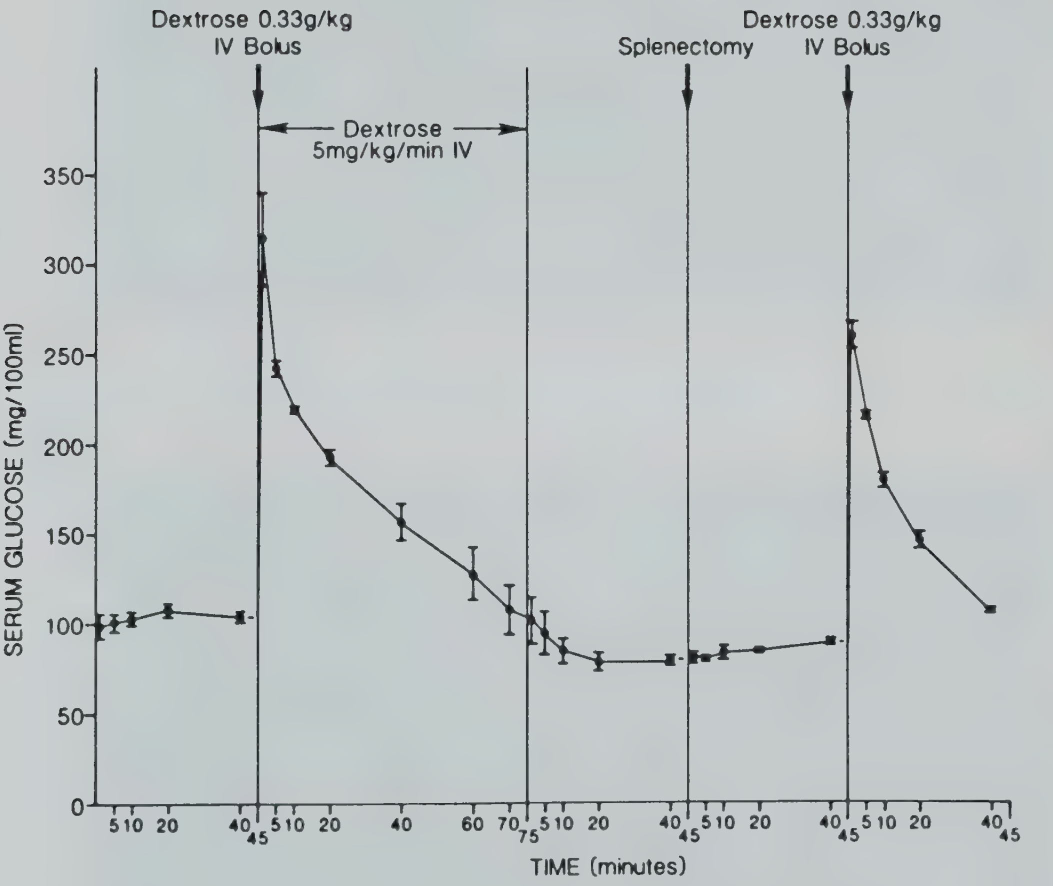


Fig. 14 Peripheral venous glucose after glucose challenge in normal control dogs.

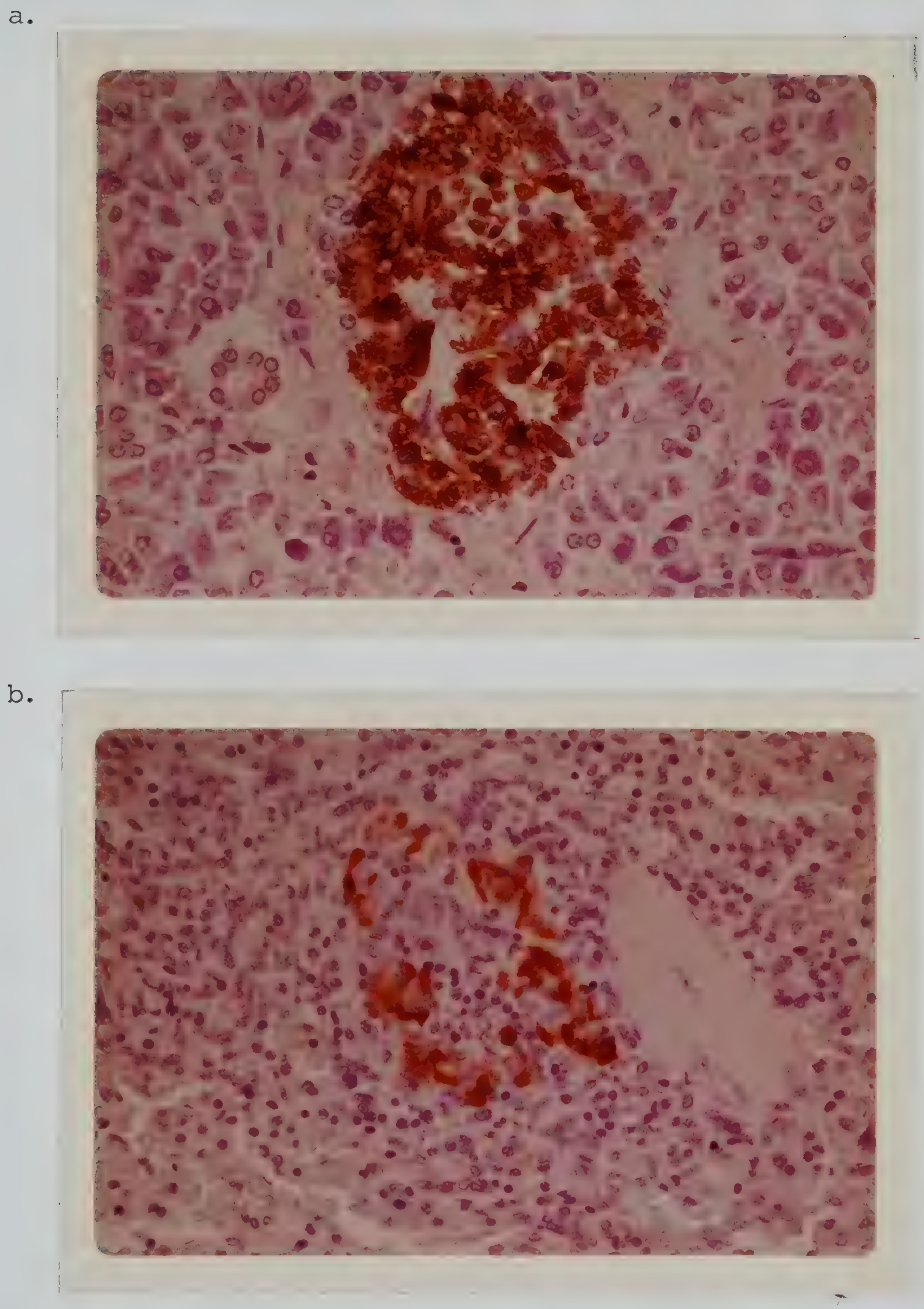


Plate 10 Immunocytochemical localization of insulin in islets. a. Normal control pancreas (x 100) b. Degranulated intrasplenic islet (x 100).

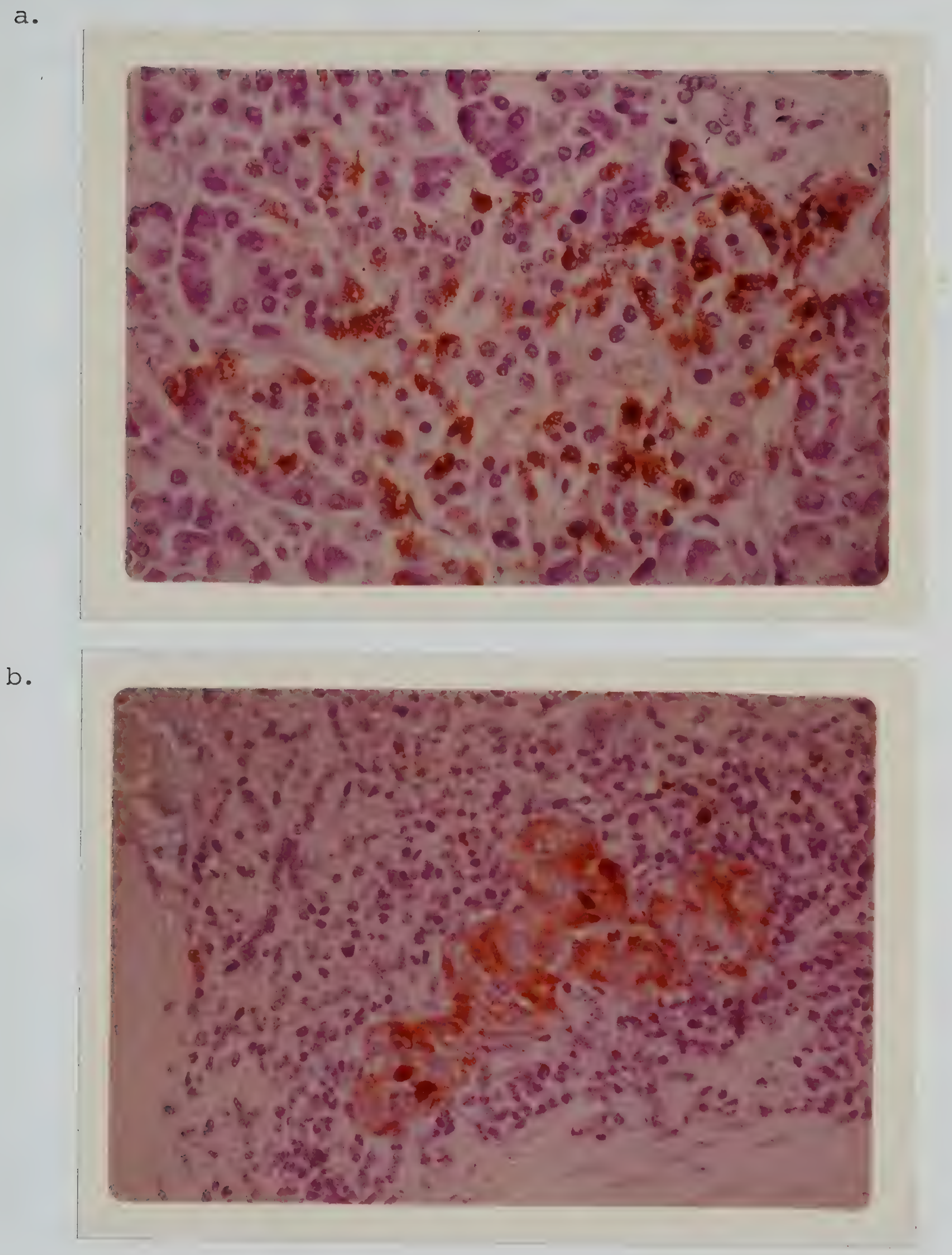


Plate 11 Immunocytochemical localization of glucagon in islets a. Normal control pancreas b. Degranulated intrasplenic islet. In transplanted islets, the peripheral rim of A cells retains its position (both x 100).

Cryopreservation (Fig. 15)

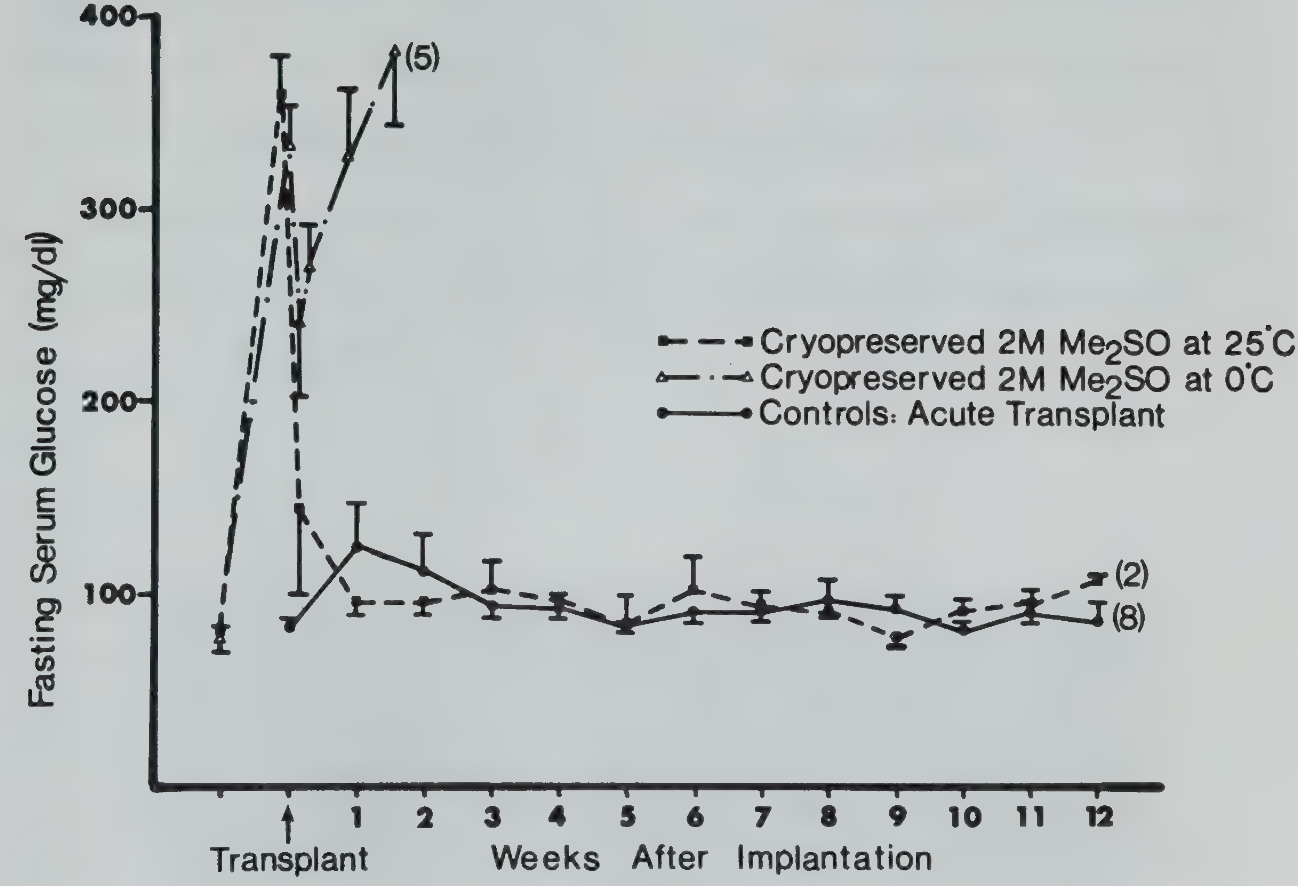


Fig. 15 Plasma glucose concentrations during the first 12 wk after implantation of autogenous islets cryopreserved after two different methods of exposure to Me_2SO.

The acutely implanted controls became normoglycemic within 5 days and this was maintained throughout follow-up. In the group implanted with grafts cryopreserved after exposure to 2 M Me_2SO at $0^{\circ}C$, there was a slight decline of glucose for 48 hr, but levels gradually increased thereafter to $\geq$ 350 mg/dl just before they were killed. In 2 dogs after exposure of the graft to 2 M Me_2SO at $25^{\circ}C$, normoglycemia was restored within 48 hr, and this has been maintained

on long-term follow-up for 3 mo. Intravenous glucose tolerance tests have been performed at 2 wk on follow-up and showed a slow decline of glucose (K = 0.8 ± 0.1%) with loss of the peak insulin response. By 2 mo, recovery has occured, as the K value is 1.95%.

Histologic studies of cryopreserved grafts reveal preservation of islets with striking degeneration of the exocrine components of the tissue (plate 12).

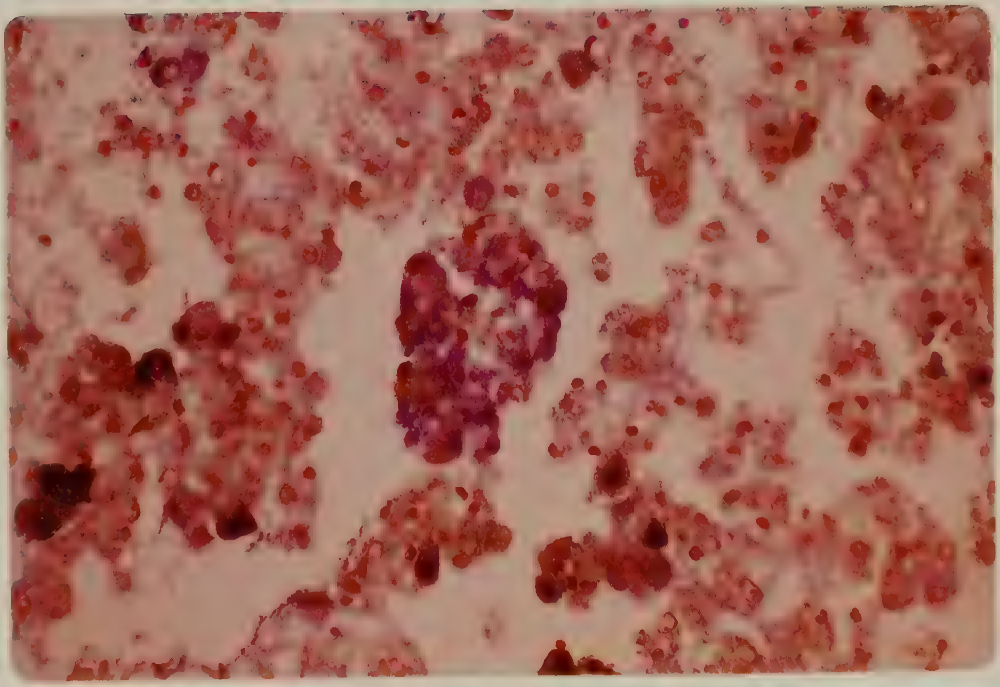


Plate 12 Frozen-thawed graft. Islet is well preserved, acinar tissue has degenerated into "ghost" cells (Gomori's aldehyde fuchsin x 100).

V DISCUSSION

In the quest for a cure of diabetes mellitus by pancreatic islet transplantation, techniques for islet isolation, implantation, preservation and immune alteration have been largely successful in streptozotocin-diabetic rats. The efficacy of these techniques has been verified by metabolic studies. Though many excellent studies have extended these techniques to large-mammal and clinical islet transplantation, success has been sporadic. The present research, using autotransplantation of islet-containing pancreatic tissue in dogs, has explored a method for islet isolation, optimal implantation techniques and cryopreservation of grafts in large mammals. This has permitted a definition of metabolic efficiency of islet grafts in these species. The results of the experiments even have some indirect benefit for ameliorating the greatest problem of all: allograft rejection.

i. Isolation techniques

Inadequate harvest of insufficient viable islets from single pancreases is an important problem, as this has precluded a detailed analysis of other aspects. Even with rat experiments, the underlying theme has been inadequate islet harvest from single pancreases. For example Ballinger and Lacy (43), reporting the first successful transplantation of pancreatic islets in rats, isolated about 200 islets (of a possible 5,000-10,000) from a single pancreas. This problem was circumvented by collecting 600 islets from multiple donors for one recipient. In large mammals, the problem was compounded because

the compact nature of the pancreas dictated isolation of even less islets. Experiments with monkeys and pigs failed (46). The use of fetal and neonatal tissue with its high relative composition of islet tissue did not ameliorate the problem.

The breakthrough came in 1976, when Mirkovitch and Campiche reported that islet separation from exocrine tissue was not necessary for successful transplantation in dogs (77). Since then, islet-containing tissue has been used successfully in several research laboratories for large-mammals (21,32,61). Newer approaches have been taken to further increase yields from single pancreases: collagenase perfusion via pancreatic ducts: cell separation and reaggregation as pseudo-islets; venous disruption of pancreases (28,39,71,79).

In the present study, we have combined the principle of not separating islets from exocrine tissue with collagenase perfusion via ducts. Using a modification of Horaguchi and Merrell's method of isolation (we deleted the steps of extra trypsin digestion), we isolated 24% of the B cell mass from single pancreases. Though lower than the yields from Horaguchi and Merrell's work (57%), this compares favorably with yields of 8% obtained by Kretschmer _et al_ (29). Nevertheless, even though islet yields seem low, they are sufficient to prevent diabetes after total pancreatectomy in dogs. Further, fasting normoglycemia (BG < 150 mg/dl) is restored within 4-5 days of implantation in 9 of 11 dogs, and is maintained during 3-5 mo of follow-up in 6 dogs. Only 1 dog became diabetic on follow-up, and that was after sepsis arising from a necrotizing infection developing in a venepuncture site 6 wk after implantation.

The importance of these findings is that using these techniques, we were able to reproduce a technique for isolating sufficient viable islets from a single pancreas to ameliorate diabetes. As pointed out by Mehigan _et al_ (61), a standardized method must exist which must be reproducible in several centres if pancreatic islet transplantation is to become a reality. Indeed, experience in our own lab has confirmed that it is very difficult to reproduce a reliable method for preparing islet grafts. Lack of reproducibility brings basic research studies to a halt, denies their extension to clinical islet transplantation, and prevents extension to islet isolation from the glands of chronic pancreatitis.

An important sacrifice is apparent in this method of graft preparation: there is significant contamination with exocrine cells. In this study, no attempt was made to purify the B cells of their exocrine contaminants. Horaguchi and Merrell obtained a 6-fold purification, (28) though they digested the tissue further with trypsin before implantation. The hazards of this lack of purification are well known and include D.I.C. and portal hypertension due to release of tissue thromboplastins. Further, the exocrine component may continue to digest the graft due to release of digestive enzymes from the damaged cells. These hazards may be circumvented by choosing the correct site of implantation (spleen, via splenic veins) and possibly by new purification techniques (cryopreservation). These will be discussed later.

In summary, preparation of islet-containing tissue by collagenase perfusion of pancreatic ducts is a readily reproducible method for

isolating sufficient islets from a single large-mammal pancreas to reverse diabetes in a single recipient. Perhaps the door is now ajar for a standardized method of graft preparation from large-mammal pancreases.

ii. Optimal implantation methods

The three critical factors to consider in choosing an optimal implantation site have been summarized by Kemp et al (44):

1. maximum survival and function of transplanted islets
2. maximum effectiveness of secreted hormones
3. safety to the patient

Early experiments with large mammals failed largely because subcutaneous sites for islet implantation did not allow sufficient vascularization of islet tissue (37,38). Even Ballinger and Lacy, who obtained sufficient islets from multiple rat pancreases to ameliorate diabetes, did not completely normalize endocrine function in streptozotocin-diabetic rats (43). They had used an intraperitoneal implantation site. Kemp (44), extending these studies, found that an intraportal route of transplantation was superior to intraperitoneal and subcutaneous sites of implantation in rats. He found that, for a given number of islets, the portal vein route permitted more complete normalization of endocrine function compared with the other sites. Thus it appears that immediate vascularization by direct access to a vascular bed is critical to early survival of islets. Quantitative studies of rat islets embolized to portal veins have shown that only about 50% of islets survive (78).

In large mammals, enthusiasm for the intraportal route of implantation was tempered by the discovery that islet preparation from exocrine tissue was unnecessary (77). Such grafts, rich in tissue thromboplastins, enzymes and vasoactive substances, cause portal hypertension (30). This is due to coagulation in portal venous radicles induced by tissue thromboplastins, portal venospasm from vasoactive peptides, and mechanical obstruction of venous radicles. In addition, disseminated intravascular coagulation, induced by tissue thromboplastins, appears to be a problem (80).

A useful alternative to the intraportal route appears to be the spleen (29,77). This site is well vascularized, and appears to tolerate enzymes and contaminants well, possibly because the rapid sinusoidal blood flow dilutes these products. The result is less autolysis of islet tissue locally, and presentation of lower concentrations of thromboplastins and enzymes to the liver. Though one study showed no advantage of intrasplenic over intraportal transplantation (101) the splenic route has come to be favored.

The technique for intrasplenic implantation has been similar in several research laboratories. The catheter which conducts the graft has been advanced through the splenic pulp either by direct stab of the pulp (20,61) or by threading it into the pulp after entry of a splenic vein (77). This technique of direct injection into the pulp was necessary because of the size of the pancreas fragments (which has been described as 2-4 mm, or "ability to pass through 15-16 gauge needles"). Thus, these particles were too large to embolize into a vascular bed.

Direct injection into splenic pulp, while successful in several studies, ignores 2 of the critical factors to be considered in implantation. First, the technique is traumatic. Mirkovitch and Campiche and Kretschmer <u>et</u> <u>al</u> have reported splenic rupture after implantation in dogs (59,60). Second, direct injection into the pulp necessitates a breach of the vascular space, and tissue is deposited in a dead space remote from the vascular compartment. In this dead space, the exocrine enzymes, rather than being washed away, remain localized and autolyse endocrine tissue. Further, the pancreas fragments lie adjacent to each other, rather than bathing in the nutrient-rich plasma which supplies their life through nutrient diffusion.

In the present study, we compared reflux of islet tissue into splenic veins (SV) with deliberate implantation directly into the splenic pulp (SP) to see if a less traumatic and metabolically efficient implantation could be obtained. This was made possible because the tissue fragments, $\leq 400\mu$, passed easily through 22-gauge cannulas inserted in splenic veins. In 9 of 11 SV dogs, the findings of normoglycemia (BG < 150) beginning 5 days after implantation and extending for 6 wk, are in marked contrast to hyperglycemia (BG > 250) throughout the 6 wk in 5 SP dogs ($p < 0.001$). Thus, direct access to the vascular bed of the spleen is more efficient than direct implantation into the pulp. The reason for this is reflected by the histologic findings: SV spleens at 5 mo showed prominent islets with atrophied acinar tissue; SP spleens at 6 wk showed fibrosis, abundant acinar tissue with zymogen granules and no islets. Direct implantation appears to leave exocrine tissue in a position where it maintains

viability and perhaps the enzymes released continue to autolyse islets. In the 2 unsuccessful SV dogs, the cannulas had passed out of the vein into the splenic pulp, thus the graft was not as widely dispersed and likely was similar to SP dogs. The SV technique was equally as safe as SP injection, as no cases of DIC, portal hypertension or splenic rupture occurred. In fact, it was safer, since the splenic pulp was not subjected to the hazards of blind pucture: 2 spleens in the SP group required prolonged compression over the site of capsular injury to control troublesome oozing.

Before summarizing, important factors are noteworthy about reflux via splenic veins. First, after releasing vascular clamps, islet tissue could be seen embolizing to the liver. Though no other clinical nor necropsy evidence of portal hypertension was present, the possibility of this must be borne in mind. Portal hypertension following inadvertent embolization of intrasplenic pancreas fragments was well demonstrated by Dutoit et al (80). Second, two animals died within hours of surgery. The absence of obvious identifiable factors leading to death has raised suspicion that hyperinsulinemia with hypoglycemia and hypokalemia led to their demise. Unfortunately, serum glucose and plasma insulin were not assayed during this period, so no definite conclusion can be made. Hypoglycemia, hypokalemia and death is well documented with whole-organ allografts in dogs, and poses a hazard clinically (81). Certainly, the total access of the graft, containing many damaged cells, to the vascular bed of the spleen, coupled with a rapid sinusoidal blood flow, made hyperinsulinemia, hypoglycemia and hypokalemia a threat. Third, the spleen plays an important role in

host defence against encapsulated bacteria with phagocytosis and formation of tuftsin. Injection of pancreatic tissue may alter this function, and this may be even more important with immune suppression to maintain allografts. One dog, who had been normoglycemic for 6 weeks, became hyperglycemic when he developed sepsis from a necrotizing infection at a venipucture site and had to be killed: possibly, impaired immune serveillance played a role in sepsis. Fourth, the technique requires a completely mobile spleen and the cannulation of splenic veins is meticulous. Though no problems were encountered, there is an ever-present risk of splenic injury.

In summary, optimal conditions for implantation of islet-containing pancreas fragments include immediate access to a vascular bed. Reflux via splenic veins offers this condition, and appears to be more efficient in maintaining normoglycemia than splenic pulp injection. Further, reflux is safer than per-capsular puncture with intra-pulp injection. These conditions, together with the portal route of insulin delivery afforded by the spleen, and elimination of problems with portal hypertension suggest that the spleen is an attractive site for clinical islet transplantation (50.

When we attempted to embolize the gemisch into the portal vein, profound portal hypertension occurred with portal pressures increasing from 6 cm H_20 to > 40 cm H_20. This was accompanied by engorgement of mesenteric veins, splenomegly and darkening of the bowel, and was followed by death within hours. This suggests that the gemisch has a very high contamination with thromboplastins, vasoactive amines and enzymes and that slow infusion and pharmacologic agents (trasylol and

and heparin) were unable to prevent deleterious effects. In contrast, the remarkable success with splenic sites indicates that the spleen is a safer site for injection and can buffer these contaminates well.

In the 2 dogs in whom the gemisch was embolized via short gastric arteries to the rich capillary arterial bed of the stomach submucosa, success was denied. Even before their abdomens were closed, arterial pulsations had ceased in the remaining short gastric arteries and the stomach was blanched. Profound hyperglycemia (BG > 300) ensued and remained until animals were killed 2 wk later. At necropsy, only fibrous nodules were identified in the stomach submucosa, and no pancreatic tissue at all was seen on l.m. It appeared that all the advantages of this richly oxygenated capillary bed were lost because capillaries thrombosed, causing the graft to infarct.

iii. Metabolic studies

It is essential to define the metabolic efficiency of implanted grafts of islet tissue. First, the ability to normalize fuel homeostasis can be viewed in perspective with other new methods of treatment (insulin pumps and whole-organ grafts) by comparing metabolic parameters. Second, an early, sensitive indicator of allograft deterioration due to rejection must be defined if immunosuppressive intervention is to be timely: only a careful definition of metabolic behaviour will enable such a subtle indicator to be found. Third, insight may be gained as to whether the techniques for islet graft preparation of implantation are optimal.

Metabolic function of whole-organ allografts has been documented

in dogs (81) and in humans (82). However, metabolic efficiency has not been well defined after implantation of free grafts of islet tissue. Further, the multitude of techniques for graft preparation has rendered comparison of metabolic function difficult.

There are 3 types of metabolic studies: glucose tolerance testing (GTT) with assay of glucose, insulin and glucagon in peripheral veins; 24 hour serum profiles of insulin, glucagon and C-peptide secretion; cannulation of veins draining the implant site with assay of the venous effluent for insulin and glucagon. Additional information can be gained by stimulating the grafts before these studies with secretogogues such as arginine, glucagon or tolbutamide. At GTT, logarithms of glucose values can be plotted on semilogarithmic paper. This produces a series of points to which a straight line may be fitted using the method of least-squares. The slope of this line is termed the K value and expresses the percentage decline in glucose concentration per minute (72). Values are useful for a quantitative estimate of functioning B cell mass, values < 1.0% being in the insulin dependent diabetic range (26).

A further measure of metabolism of insulin is provided by determinating the flux of secreted insulin across the hepatic vascular bed, in order to determine if the extraction of secreted insulin is altered after transplantation. In the present study all of the types of metabolic study were used.

1. GTT with assay of glucose, insulin and glucagon in peripheral veins.

In this study, the rate of glucose decline was slower after

implantation, with mean preoperative K values of 3.4 ± 0.2% being reduced to 1.4 ± 0.1% at 1 mo post-operative. This 50% reduction was also obtained by Kretschmer (32), who showed a decrease from 3.3% to 1.6%. Mehigan et al obtained postoperative values of 1.76%. Our results, though lower than these others, compare favorably with them. All values are well above the insulin-dependent range.

Insulin levels during GTT, which peaked at 55 ± 6 μU/ml preoperatively, were reduced to 12 ± 2 μU/ml at 1 mo after implantation. Reduction was also reported by Mirkovitch and Campiche (59), the first to report pancreatic fragment implantation in dogs: they reported a 50% reduction of the preoperative values. This blunted peak insulin response has been confirmed by others (21,60) as well. Schulak hypothesized that this represented impaired insulin release due to collagenase - induced damage to the islets (21). It has been shown that excessive exposure to collagenase can cause impaired insulin release in the splenic venous effluent after intrasplenic implantation (37). Further, it has been shown that trypsin, a component of the more impure forms of collagenase, can cause impaired insulin release from B cells, possibly by damaging their glucoreceptors (53). However, in our studies, we used a purer form of collagenase, devoid of trypsin, although the exposure time to collagenase (30 min) was longer than that of Kretschmer's (20 min). However, it is not possible to make firm conclusions regarding a qualitative defect in islets from a study of peripheral vein insulin levels. The efficiency of hepatic extraction of insulin (50% in one pass), dilutional effects of the systemic circulation, and errors due to the time delay from

stimulation to peripheral sampling all may have hidden a true insulin peak response. This is especially possible in this situation since the B cell mass may be reduced to 20% of the original after implantation. Thus, quantitative and qualitative defects are best studied by examining insulin levels in the splenic venous effluent, rather than the peripheral veins (60).

Fasting glucagon levels were higher 1 mo after transplantation (59 ± 12 pg/ml) than they were preoperatively (33 ± 3 pg/ml). Since the A cells which produce glucagon are at the periphery of the islet, the elevated glucagon levels suggest that collagenase - induced damage to the islets may be minimal.

During GTT, glucagon levels did not change appreciably from the basal fasting levels. Theoretically, during glucose tolerance testing, glucagon should be suppressed since the A cells are exposed to a critical increase of insulin concentration which maintains their sensitivity to shutoff of glucose (83). Though this suppression occurs after segmental gland transplantation (82), it was shown to be absent in one study after pancreatic fragment transplantation in dogs (21). However, the results of that study, together with our own (in which suppression was not even consistent preoperatively), suggest that the insulin-mediated glucose shutoff of A cells is a very weak stimulus. No firm conclusions can be drawn regarding glucagon suppressability.

Another approach similar to GTT has been intravenous tolbutamide stimulation testing. This was done by Kretschmer _et al_ (60) after intrasplenic pancreatic fragment autoimplantation. Plasma

insulin concentration increased from fasting levels of 6 μU/ml to only 10 μU/ml, in contrast with 6 μU/ml to 34 μU/ml in normal controls. However, these tests, with peripheral venous assay of insulin, are subject to the same pitfalls as GTT, and they do not differentiate qualitative from quantitative defects in insulin secretion.

One of the remarkable aspects of the GTT in these studies was the ability of the graft to maintain its efficiency with long-term follow-up. For example, 1 month data compared with 2-3 mo data showed: K 1.4% and 1.5%; insulin peak response 12 ± 1 μU/ml and 11 ± 1 μU/ml; glucagon showed no change from baseline.

This indicates that the reduced B cell mass was able to withstand the entire burden of fuel homeostasis without degenerating with time. A slow decline in graft function has been observed in patients who had pancreatic islet transplantation after near total pancreatectomy for chronic pancreatitis (84). This may be due to exhaustion of a marginally adequate islet graft. Certainly, morphologic evidence raises this possibility in our own studies. Immunocytochemical localization of insulin and glucagon in the transplanted islets suggested degranulation compared with controls. Though this apparent degranulation was evident, after 3-5 mo follow-up it should be noted that the islets were studied after cannulation studies in which they were stimulated with glucose.

One of the reasons why transplanted islet cells degranulate, and grafts fail after prolonged follow-up may be related to denervation. Bewick (81) showed that portal hyperinsulinemia resulted after autonomic nerves to the pancreas were sectioned. He concluded that

the nerves, through a tonic inhibition of islets, prevented indiscriminate release of insulin. Certainly, the loss of this "brake" on insulin secretion may be reflected by islet degranulation.

2. Profiles of fasting insulin and glucagon.

In these studies fasting levels of insulin ranged from 2-14 μU/ml on daily monitoring. These levels were erratic and in some cases were higher than before pancreatectomy; in some they were lower. Further, the levels did not bear a relationship on concurrent blood glucose levels. These findings have been noted previously (59). This indicates that insulin levels are not a useful guide to graft function, using these techniques of graft preparation and implantation. The efficient hepatic extraction mechanism and the effects of dilution in peripheral blood appear to reduce insulin concentrations so as to render their interpretation difficult. Compared with pancreas grafts draining their venous effluent systemically, this is a disadvantage. In such grafts, peripheral hyperinsulinemia results because the hepatic extraction mechanism is bypassed and the pancreas is denervated. This permits a 'window', via systemic blood assays, on the insulin secretion and hence the function of B cells. In the early phases of rejection, the hyperinsulinemia is aggravated (85) providing a sensitive warning signal of impending graft loss.

Glucagon levels, like insulin, proved to be erratic when measured in fasting blood collections, thus rendering them unreliable in following graft function. Part of the problem was due to the glucagon assay, which does not differentiate glucagon of islet-cell origin from the glucagon-like activity secreted by gut cells.

There is one way to circumvent the problems associated with measurement of insulin levels after transplantation: that is, to measure C-peptide. Though not measured in this study, this deserves comment. C-peptide is the polypeptide segment devoid of amino and carboxy-terminal residues which is cleaved from proinsulin to form insulin (86). This process, which occurs within B cells, produces equimolar concentrations of insulin and C-peptide since they are derived from one molecule of insulin. Equimolar quantities of each are secreted, and C-peptide, unlike insulin, is not cleared by the liver but is secreted in the urine. This provides a sensitive marker for B cell function. The practical advantage over insulin assay is evident: hepatic clearance will not alter the peripheral vein concentrations of this peptide; further, exogenous insulin and circulating insulin antibodies will not affect this index of graft function. Urinary C-peptide has proved to be a very useful guide to immunosuppressive requirements after pancreatic segment transplantation (82).

3. Cannulation Studies

In these studies, mean basal insulin levels in the splenic venous effluent of 5 dogs after splenic vein cannulation were 27 ± 5 μU/ml. This increased to 59 ± 11 μU/ml after glucose stimulation, approximately a 2 fold increase in output. In previous studies the basal levels of insulin, 34 μU/ml (57) and 60 μU/ml (49), doubled after glucose challenge to 77 μU/ml and 130 μU/ml, respectively. Though these figures show an ability of islets to respond to an acute change in glucose concentration, they do not address the question of whether the islets are qualitatively intact. To study this, kinetic studies of

insulin release are necessary.

Kinetic studies of insulin release from islets in vitro, have shown that they secrete insulin in a biphasic pattern after glucose stimulation (87). The first phase, arising within 50-100 seconds after glucose challenge, peaks after that latent period, then returns to a basal level after 5-10 min. Then, if glucose stimulation continues (as might occur as a meal is being absorbed over a period of an hour), a second phase of insulin release occurs. During this second phase, insulin levels continue to rise until the glucose stimulus is stopped. It has been suggested that the first phase is due to the sudden release of insulin from a labile compartment of stored insulin; the second phase is due to a gradual release of newly-synthesized insulin (87).

This pattern of secretion has been shown in vivo after implantation of neonatal rat pancreases under the kidney capsule of alloxan-diabetic rats (88). In that study, there was a marked increase of insulin within the first minute, and a second rise occurred from 5 min onward.

In our studies, the biphasic response was confirmed. After glucose challenge, in 5 dogs, insulin peaked at 55 ± 13 μU/ml at 1 min. The challenge was maintained as a constant infusion and after 10 min, insulin levels gradually rose, reaching a second peak of 77 ± 15 μU/ml 60 min after the infusion began. This suggests that the glucoreceptors and mechanisms for release of insulin are intact in dog islets 3-5 mo after intrasplenic implantation.

The importance of the preservation of this biphasic response must

be emphasized. When first challenged with glucose, an islet should promptly release a large quantity of stored insulin to avoid an exaggerated increase of serum glucose, and this is reflected by the first peak of secretion. As glucose is constantly infused, insulin secretion must rise to meet the new steady-state of increased glycemia, and this is reflected by a second peak of secretion. Here, we see that the transplanted B cells maintain their "fine tuning" role in attempting to prevent wide excursions in blood glucose levels. These wide excursions, implicated in the pathogenesis of microvascular complications in diabetes even with conventional methods of insulin therapy, may now be appropriately reduced to the narrow range seen in normal individuals.

Though these studies suggest that the transplanted islets maintain their "fine tuning" role in response to glucose challenge, it remains to be seen how they function in alimentary gluco-regulation. In particular, how does denervation affect the islets' response to fuels, and how do the islets repond to stimuli other than glucose? Recently, a study has addressed these questions (88). Oral glucose tolerance tests were performed in alloxan-diabetic rats who were cured by implantation of neonatal pancreases under their kidney capsules. After the carbohydrate-rich meals in control rats, plasma insulin increased by 200% 1-2 min before plasma glucose rose. This anticipatory response to a glucose increase after a meal was lost after pancreas transplantation, consequently serum glucose rose in an exaggerated fashion. The investigators concluded that this was due to vagal denervation of the pancreas fragments. Whether this was mediated by direct absence of cholinergic receptors in the islets, or via loss of vagal-mediated

release of enteric peptides was not clear. However, these studies show that an important regulator of the fine control of blood glucose is lost after transplantation of islets.

Insulin levels in transplanted dogs were lower than those obtained in normal control dogs. Several explanations are offered. First, there is a quantitative reduction in the B cell mass after transplantation. Only 40% of B cells from single pancreases were harvested and if half of these survive in the vascular bed of the implant site, only 20% of the original mass remains. Second, it is possible that exposure to collagenase damages the ability of islets to respond to glucose stimulation. Gray _et al_, who studied insulin and glucagon release from intrasplenic islets after arginine stimulation, showed greater insulin release when collagenase was not used to prepare grafts (89). Third, transplanted dogs had lost weight and showed some evidence for malabsorption (despite pancrealipase replacement). Two dogs were anemic. This may have led to a reduced ability to withstand the rigors of the long experiment with greater catecholamine responses to the stress than control dogs. This may have blunted insulin release more. Fourth, because of some evidence for malabsorption, B cells may have been less responsive to glucose stimulation. It has been shown that fasting for 24 hrs, by decreasing cyclic AMP within B cells, reduces their responsiveness to glucose challenge. Malabsorption would aggravate this.

Glucagon levels in the portal venous effluent did not show suppression after glucose infusion in either control dogs or transplanted dogs. Once again, conclusions regarding A-cell function are difficult

to make. It appears that the shutoff of A cells by glucose is a very weak response, thus equivocal results are obtained. A further factor rendering interpretation difficult is the stress-induced catecholamine release under the anesthetic and surgical conditions. This may have induced an excessive release of glucagon from A cells, blunting any suppressive response to glucose. This was quite apparent in 1 dog in which hypovolemia occurred during the fourth hour of study. Glucagon levels rose markedly from 392 pg/ml (baseline after cannulation) to > 1000 pg/ml. It is interesting to note that this was accompanied by a rise in insulin to 580 μU/ml. Two observations may be made. First, this stress situation suggests that the transplanted islets respond to a stress situation by increasing glucagon release, with a view to increasing glucose levels through gluconeogenesis. Second, the rise in insulin suggests that receptors on the B cells responded appropriately to glucagon stimulation by increasing insulin release.

The cannulation studies reported have also enabled us to satisfy the classic requirements of endocrine tissue transplantation. First, we proved that the spleen was the site of implantation by demonstrating the release of insulin from this site after glucose stimulation. That insulin levels did not peak in the hepatic veins made it unlikely that the liver harboured a significant number of islets which had embolized to that site. Histologic proof of intrasplenic islets was confirmed after the splenectomy was performed in the fourth period of study. Second, after splenectomy, an endocrine deficiency state resumed. Glucose decline followed a diabetic curve (0.7 ± 0.1%). Though this could be criticized since the stress of surgery causes insulin resis-

tance, it is evident that a true deficiency state developed: insulin levels dropped below 4 μU/ml (the limit of assay sensitivity is 4 μU/ml); glucagon levels in portal veins dropped precipitously from 220 ± 49 pg/ml to 37 ± 3 pg/ml at the conclusion of the study.

In summary, metabolic studies show that pancreatic islet transplantation can normalize, via the portal route, fuel homeostasis sufficient to maintain tolerance to intravenous glucose. During glucose challenge, the insulin release is biphasic, indicating that the "fine tuning" mechanism for preventing wide fluctuations in blood glucose is intact. The efficiency of these grafts does not falter with prolonged follow-up.

iv Cryopreservation

Isolation of sufficient islets of Langerhans will create a demand for methods of graft preservation until transplantation, as for other organ transplants. Short term preservation is essential to permit time for histocompatability testing, and permit transport of pancreas grafts from the location of the donor to that of the recipient. Long-term preservation would enable islets to be stored in a tissue bank and permit better histocompatability matching. Three methods of preservation have been tested: tissue culture; cold storage; cryopreservation.

Tissue culture enables rodent islets to be stored at least 1 week, and the islets are capable of responding in vitro to glucose and restore normoglycemia in diabetic recipients (78). Culture not only provides a method for graft storage, but also improves quality of grafts by: enabling metabolic recovery of islets after isolation (90, 91); purifying fragments of islet-containing tissue by allowing deple-

tion of exocrine enzymes and cells (92,93); and appears to reduce immunogenicity of allografts by destroying passenger leucocytes trapped within the graft (94). However, the sophisticated equipment and fastidious conditions required for tissue culture lessens the practicality of this method for long-term maintenance of large-mammal islets.

Cold storage, routinely used for 24-48 h for renal grafts before transplantation, has also been studied for islets. Payne demonstrated in vitro viability of canine islets stored for 48 h at room temperature (95). Schulak tested viability of canine islets stored for 24-48 h at 4°C by transplanting the islets (21); and found that 24 h was optimal, but after 48 hr, grafts deteriorated.

It also appears feasible that cold storage may help purify grafts of islet containing fragments. Though culture studies have not been done to assess this, it has been shown that exocrine tissue is very sensitive to cold conditions, hence it is destroyed (54,84).

A new approach to graft storage has been shown by Toledo-Pereyra (96). Canine pancreases were removed then perfused with cryoprecipitated plasma supplemented with salt-poor albumin at 5-7° for up to 48 hr. Then pancreases were prepared into grafts of islet tissue and autoimplanted resulting in prolonged survival. This approach would permit pancreases to be transported between hospitals where the donor and recipient were separated and permit transfer of the pancreas to an islet cell lab for processing before transplantation.

Though feasible for short term storage of 24 hr, hypothermia does not appear to offer promise for long-term storage, as graft function

deteriorates after 48 hr.

The pitfalls with tissue culture and hypothermia have provided the stimulus for a third method of graft storage: cryopreservation. Transplantation of cryopreserved islets of Langerhans can render diabetic rats of the same inbred strain clinically normal (31). However, studies of cryopreservation in large mammals and man have been limited, mainly because the inability to collect sufficient islets from single pancreases has made interpretation of viability a difficult task.

In the present studies, sufficient islets were isolated from single dog pancreases to permit a study of cryopreservation. In the initial studies, freezing and thawing protocols which were found optimal for rat islet viability were used (31). Five recipients of autogenous pancreatic fragments cryopreserved in this manner became hyperglycemic and rapidly lost weight. Though blood glucose was transiently reduced for 48 hr, this could not be regarded as graft function since dying B cells may have released insulin during that period. In one of these dogs, blood glucose was transiently reduced to 150-200 mg/dl between 2 and 4 wk, then hyperglycemia rapidly resumed. This failure was in striking contrast to the success of implanting fresh islets by using the same implantation techniques and indicates some of the problems involved in extending cryopreservation techniques to large mammals. First, the species-related variations in islet tissue may alter freezing rates, permeation of cryoprotectant into cells, thawing rates and osmotic shock during removal of the cryoprotectant (97). Second, the size and nature of the tissue, in this case fragments of islet-containing pancreas rather than isolated islets, may influence the

degree of permeation of the cryoprotectant into the cells (97).

We modified the freezing technique by exposing the tissue to 2 M dimethylsulfoxide (Me_2SO) at 25°C for 15 min instead of 0°C. This was reflected by normoglycemia within days of implantation in both dogs in whom the modification was used. Further, intravenous glucose tolerance testing at 2 wk showed an appropriate decline in glucose, and prolonged normoglycemia has been present in one for 3 mo at this writing.

In view of this success, some comments are necessary. The permeation of Me_2SO into cells is temperature-dependent, increased temperature resulting in greater permeation (98). Thus, it appeared that greater permeation of Me_2SO into islet cells occurred, protecting the cells from damage due to intracellular ice-crystal formation. Whether the difference from rat islets is due to species-related differences in permeability to Me_2SO, or due to the presence of exocrine contaminants which increased particle size (hence increasing the distance Me_2SO had to permeate) is not clear.

In our studies, we used a storage period at -196°C for 48 hr. This was chosen simply because it proved convenient to re-implant the frozen-thawed tissue 48 hr after total pancreatectomy without subjecting the dogs to prolonged insulin therapy. However, the viability of islets, once stored at -196°C, is independent of the time for storage, and longer periods of storage are feasible. For example, Rajotte _et al_ (31) demonstrated success after transplantation of rat islets cryopreserved for 2-5 wk. Bank _et al_ (98) demonstrated _in vitro_ viability of rat islets after 1 1/2 years of storage at -196°C.

There is histologic evidence that cryopreservation enables purification of pancreas islets from exocrine tissue. Under light microscopy, exocrine cells had degenerated, vacuolated cytoplasm with disrupted membranes; islets were remarkably preserved. Though this observation was not supported by viability assays, this has been shown. Brown et al found that after cryopreservation of human fetal pancreas fragments, there was only 50% survival, as judged by reduced ability of the tissue to incorporate ^{3}H-labelled amino acids (99). Since the majority of cells in the pancreas are exocrine, the survival test essentially indicated exocrine cell survival. The possibility that cold conditions can purify pancreas tissue of the exocrine components is further supported by the results of Hinshaw et al (54,84). This group found that simply exposing human and rodent pancreas fragments to cold conditions during graft preparation could purify the islets because the exocrine tissue was so sensitive to cold.

A question which arises is whether a period of tissue culture is necessary for 48 hr after cryopreservation to enhance metabolic recovery of grafts before transplantation. The benefits have been shown by Rajotte et al (91). In our studies, though the numbers are very small, grafts appear to function well immediately after implantation. Perhaps the method of implanting (by reflux into a vascular bed) is equally effective as culture since the fragments are in immediate contact with the blood's nutrients under ideal conditions of temperature and oxygenation.

Would cryopreservation be of value in reducing the immunogenicity of pancreatic grafts, as for tissue culture? If passenger leucocytes

have freezing and thawing characteristics different from islet cells, it is reasonable to expect that these harbringers of allograft rejection would be depleted. Certainly techniques for freezing lymphocytes have been unsatisfactory when applied to islet cryopreservation (100). Results of experiments with canine allografts cryopreserved for 24 hr have indicated that the frozen islets were as effective as fresh islets after anti-lymphocyte globulin pretreatment of pancreas fragments (100).

In summary, autoimplantation of canine islets which have been cryopreserved for 48 hr is capable of rendering totally pancreatectomized dogs normoglycemic for prolonged periods of time. The optimal conditions for freezing and thawing islet-containing fragments are being established. aside from the major benefit of prolonged storage, it is also possible that freezing purifies pancreatic islet by destroying exocrine contaminants. This suggests that cryopreservation may assert itself as the method of choice for large mammal islet preservation.

VI CONCLUSIONS

Sufficient viable islets of Langerhans can be harvested from a single large-mammal pancreas to prevent diabetes mellitus in a single recipient. To ensure this, the principle of collagenase perfusion via the pancreatic ducts proved to be a critical concept. It enabled us to readily harvest sufficient islets and thus confirm another investigator's methods, a difficult task with pancreatic islet harvest techniques. The implications are: widespread success in clinical pancreatic islet transplantation will depend upon a standardized method for harvesting the islets, and this appears to be closer to reality. Clinical and basic research into other aspects of islet transplantation, such as allograft rejection, implantation techniques and graft preservation, will be made easier to interpret: greater islet yields will prevent graft malfunction due to insufficient islets from clouding the results of these other experiments.

The pancreatic fragments produced in these experiments were amenable to reflux via splenic veins during implantation. This was apparently safer than other methods of implantation. No DIC or portal hypertension occurred, as for portal embolization of pancreas fragments; the technique for implantation was less traumatic than direct per-capsular stab of the splenic pulp. These may make the spleen more attractive as a clinical site for implantation.

The implanted intrasplenic islets remained metabolically efficient for a prolonged period of time, providing evidence that islet fatigue or failure did not occur under the burden of their new environment.

Further, the efficiency of the islets, at least to carbohydrate challenge, appears to be preserved to a sensitive degree. There was an appropriate insulin response to acute as well as more sustained pertubations in glucose concentration. Here, we begin to see that pancreatic islet transplantation realizes an important objective: wide fluctuations in glucose concentration, as occur with current methods of insulin therapy, can be reduced to the narrow range present in normal individuals.

Thus, one of the major factors implicated in the pathogenosis of the microvascular complications of diabetes mellitus may be eliminated.

Optimal methods for cryopreservation of large mammal pancreatic fragments are being established. Thus, the possibility for long-term storage of viable pancreatic islets is one step closer to the establishment of a tissue bank for storage of human pancreatic islets. Further, there is compelling evidence that these techniques, by virtue of an increased sensitivity of the exocrine tissue to cold induced damage, may help to purify grafts of the unwanted exocrine tissue. This would enhance the safety of implanting islet-containing fragments.

Does the current research contribute anything to solving the greatest problem of all-allograft rejection? It does so in several ways. First, by harvesting sufficient islets from one donor to prevent diabetes in one recipient, we may eliminate the gross histocompatability problems inherent in transplanting 'pooled' islets from multiple donors. Second, the establishment of favorable conditions for islet harvest and implantation in autografts will permit more valid conclusions about longevity of allografts, why they are rejected, and enable

us to define metabolic indicators of impending rejection. Third, cryopreservation will permit time for more careful histocompatability testing and enable shipment of grafts to the location of the recipient most likely to be histocompatable. Further, by virtue of exocrine - induced injury, freezing will reduce the burden of allogeneic tissue implanted.

In summary, the problems for harvesting, transplanting and storing islets of Langerhans in large mammals are not insurmountable. Pancreatic islet transplantation thus has a favorable outlook for the future. It is possible to normalize fuel homeostasis via the portal route with minimal morbidity. The complicated technology necessitated by insulin pumps is eliminated. In contrast to whole organ grafts, the problems with thrombosis of arterial and venous anastomosis and exocrine secretions are eliminated. Further, free grafts of islet tissue offer less immunological hazard since the volume of tissue is less, and they are more amenable to immunoalteration.

Eventual clinical applications for pancreatic transplantation are apparent. First, autotransplantation after total or near total pancreatectomy necessitated by chronic pancreatitis. Second, allotransplantation in diabetics with end-stage renal disease who are already committed to immunosuppression with a renal allograft. It is hoped that the current research will establish the foundation for these clinical applications of pancreatic islet transplantation, in the quest for methods to prevent or reverse the devastating complications of diabetes mellitus.

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